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14. ABSTRACT

Treatments for lower urinary tract dysfunctions, due to spinal cord injury (SCI), are typically ineffective or have unacceptable side effects. Our project focused on identifying new therapeutic options to treat SCI-induced afferent sensitization and bladder overactivity using a T8-T9 spinal cord transected mouse model. These included phosphodiesterase type-5 (PDE5) inhibitors, β3-adrenoceptor agonists, botulinum neurotoxin type-A (BTX-A), tibial nerve stimulation (TNS) neuromodulation and a p75 neurotrophin receptor antagonist. Our studies suggest that PDE5 inhibition is beneficial by increasing nitric oxide levels that uncouple interstitial cells driving bladder overactivity. They also decrease afferent nerve firing and thus help alleviate the sensory component in SCI-induced detrusor overactivity. β3-adrenoceptor agonists were effective in treating neurogenic and myogenic bladder overactivity by suppressing nociceptive c-fibers, but not stretch-sensitive Aδ-fibers. BTX-A was effective in suppressing only neurogenic bladder overactivity. TNS neuromodulation was ineffective in treating animals without an intact spinal cord (i.e., transected). The p75 receptor antagonist, LM11A-31, had the most therapeutic benefits and was effective in preventing/treating bladder overactivity, loss the urothelial barrier, detrusor hypertrophy, and development of detrusor sphincter dyssynergia (DSD).

15. SUBJECT TERMS

Lower urinary tract symptoms (LUTS), spinal cord injury (SCI), Botulinum Toxin Type A (BTX-A), β3-adrenoceptor agonists, phosphodiesterase type-5 (PED5) inhibitors, and tibial nerve stimulation (TNS) neuromodulation

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Introduction

There are an estimated 12,000 new cases of SCI every year in the United States [1], which significantly affects the quality of life of injured patients who develop paralysis and bowel and bladder pathologies. Historically, bladder dysfunction was the major cause of death in SCI patients due to renal failure, but is now managed by clean intermittent self-catheterization. Therapeutic treatments are typically not effective and have intolerable side effects making the discovery of new therapeutic targets essential.

The three specific aims of our project were:

1) Characterize the central and peripheral mechanisms responsible for spinal cord transection-induced neurogenic and myogenic detrusor overactivity.

2) Determine the roles of urothelial (UC) and interstitial cells (IC) in spinal cord transection-induced bladder overactivity.

3) Evaluate new therapies for reducing afferent sensitization and intrinsic detrusor overactivity including β3-adrenioceptor agonists, BTX-A, $P2Y_6$-receptor antagonist and TNS neuromodulation.

We proposed in aim 1 to investigate the peripheral and central neural changes that mediate bladder overactivity following SCI. These experiments utilized optical mapping techniques to determine the consequences of afferent remodeling in the trigone and the interactions between isolated UC, IC and bladder smooth muscle cells. We utilized selective delivery of genetically encoded Ca^{2+} sensors to assess the functional changes of neurons that innervate the lower urinary tract. This methodology allowed us to record and assess Ca^{2+} transients from individual neurons and their axons. Additionally, we examined the functional consequences of afferent remodeling with *in vitro* afferent nerve recordings.

In the second aim, we investigated the roles of UC and IC in spinal cord transection. *In vitro* afferent nerve firing, bladder wall imaging and immunohistochemistry were utilized for these studies.

In the third aim, we proposed to evaluate mechanisms of four therapies including 3a) β3-adrenoceptor agonists, 3b) BTX-A injections, 3c) $P2Y_6$-antagonists and 3d) TNS neuromodulation.

β3-aderenoceptors have shown promise as therapeutic targets for treating bladder overactivity. They are highly expressed throughout the human bladder and their activation relaxes detrusor smooth muscle [2, 3]. While these receptors are thought to be the main target of β3-adrenoceptor agonist therapy for bladder overactivity [4, 5], the effects of their stimulation on bladder sensory function have yet to be fully elucidated.

BTX-A has therapeutic effects on bladder dysfunction by inhibiting acetylcholine release from parasympathetic nerves. This decreases reflex contractions and inhibits neuropeptide release from afferent nerves which reduces sensory symptoms. However, BTX-A inhibition of sympathetic nerves may decrease norepinephrine release and stimulation of detrusor β3-adrenoceptors in humans to adversely affect bladder compliance.

Neuromodulation of sacral, pudendal and tibial nerves is effective in promoting continence in non-SCI patients with bladder overactivity. TNS is of particular interest as it is minimally invasive and has long-term effects that persist after cessation of treatment. However, the mechanism by which these long-term effects occur was unclear and was a focus of these studies. A controversy also existed regarding the effectiveness of TNS in patients with SCI [6]. Further understanding of the sites and mechanisms of action of neuromodulation was necessary to address this issue.

During the work on the project we also examined the role of urothelial nitric oxide and p75 receptors in SCI-induced bladder dysfunction.

We have findings suggesting that SCI-induced bladder overactivity in part is myogenic and driven by the urothelium and IC in the lamina propria. We hypothesize that nitric oxide (NO•) released from the urothelium can inhibit propagation of pacemaker activity by uncoupling gap junctions between IC. Following SCI, reduced

efficacy of urothelial NO• could allow for the increased spontaneous detrusor contractions. NO• can also inhibit sensory nerve firing and the decreased urothelial NO• may account for part of the afferent sensitization that occurs following SCI.

It is known from animal studies that SCI leads to a rapid (within hours) disruption of the urothelial permeability barrier followed by urothelial hyperplasia due to unclear mechanisms. p75 is a neurotrophin receptor which preferentially binds uncleaved pro-Nerve Growth Factor (proNGF) and pro-Brain Derived Neurotrophic Factor (proBDNF) to trigger apoptosis, while mature neurotrophins promote cell growth *via* Trk and p75 receptors. Recent studies have demonstrated that both proneurotrophins and p75 receptors are upregulated following SCI and that receptor inhibition improves motor function in mice with spinal cord contusion [7]. Since neurotrophins and their receptors are also present in the bladder, we investigated the effects of p75 receptor blockade on loss of urothelial cells, disruption of barrier function and bladder overactivity following SCI.

Key Words

β3-Adrenoceptor Agonists

Bladder Smooth Muscle

Botulinum Neurotoxin Type A

Genetically Encoded Ca^{2+} Indicators

Interstitial Cells

Lower Urinary Tract Symptoms

Neuromodulation

Optical Mapping

P75 Receptor Antagonists

Phosphodiesterase Type-5 Inhibitors

Spinal Cord Injury

Tibial Nerve Stimulation

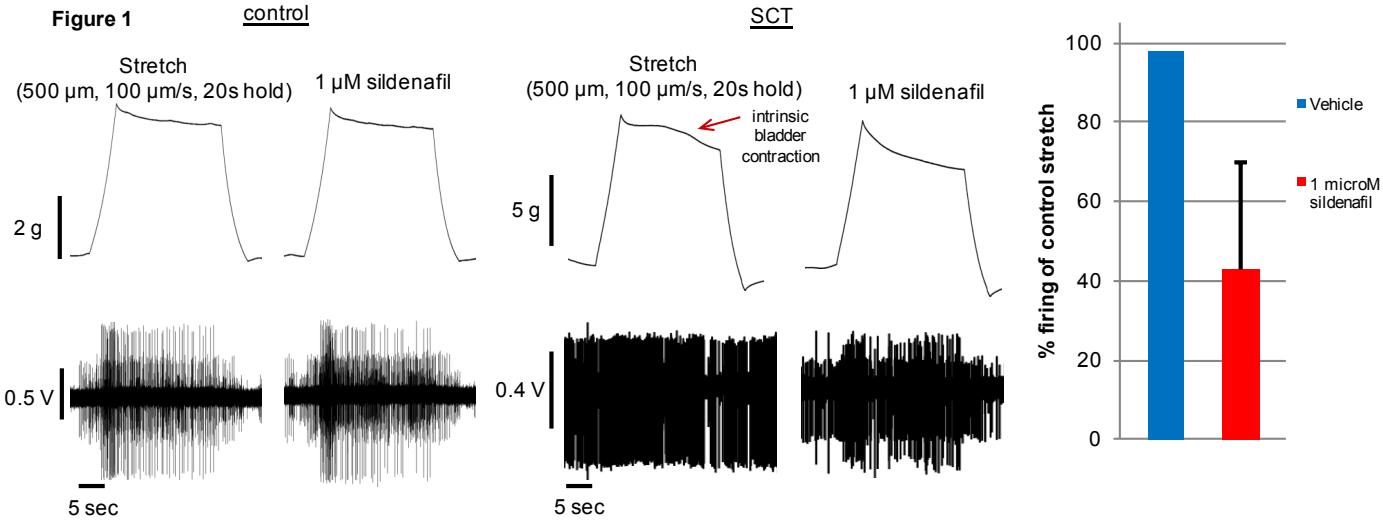
Urothelial Cells

Overall Project Summary

Central and peripheral mechanisms of DO (Aim 1):

Afferent nerve recording studies (Aim 1b):

We have evidence (see aim 2 below) that following SCI there is IC hyperplasia and gap junction coupling in the bladder such that urothelial NO• is insufficient to disrupt IC communication leading to bladder overactivity. Decreased levels of NO• may also account for increased afferent sensitization following SCI. We examined the effects of sildenafil, blocking PDE5 and thus increasing NO• signaling, on stretch-induced afferent firing in normal and spinal cord transected (T8-T9) mouse bladder sheets (**figure 1**). For these experiments, bladders

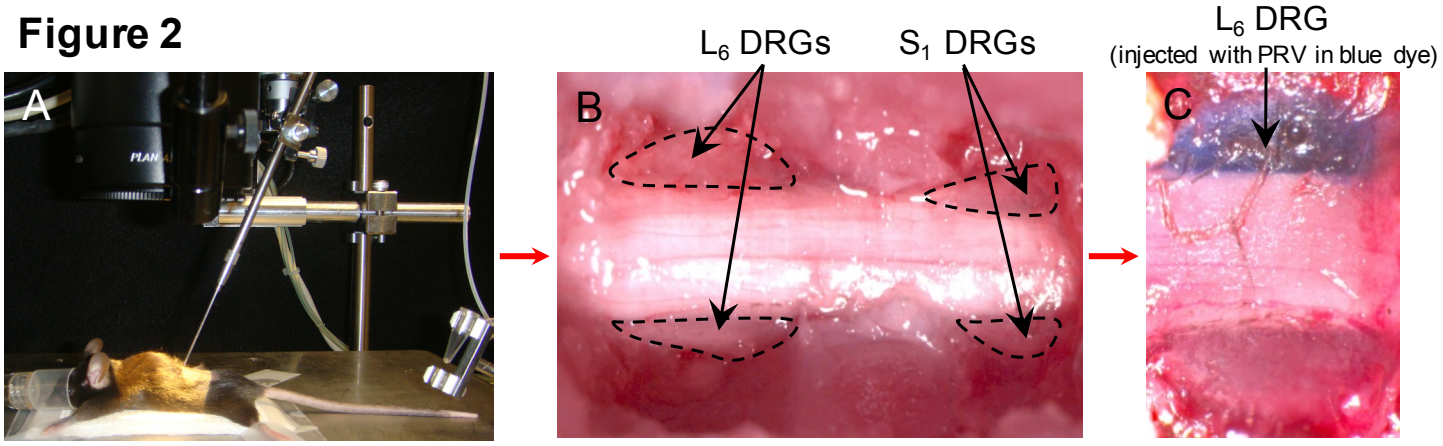


and associated pelvic nerves from L6-S2 were excised and placed in a recording chamber with oxygenated Krebs Solution. Organs were cut from outlet to dome along the midline ventral aspect to form sheets. The bladder was pinned across the midline to a fixed platform with the mucosal surface facing up and the dome connected to tension transducers. Preparations were stretched to optimal resting tension (L_o) and allowed to equilibrate for 30 min. Longitudinal stretch was applied *via* the tension transducers connected to programmable stepper motors. Afferent firing was recorded from the associated spinal roots. We have used 10 female and 10 male mice for control and 12 + 12 for SCI group. In contrast to control animals, SCI mouse bladders displayed large amplitude intrinsic contractions that stimulated afferent firing. Stretch-evoked afferent firing rates were also increased in comparison to control bladders. Sildenafil (Viagra®, 1 µM) reduced afferent firing rates (42.6 ± 27.2 % of control stretches) in response to spontaneous contractions and bladder stretches in SCI but not control mouse bladders. Contractile activity and baseline tension were also not altered by PDE5 inhibition. These data suggest that sensitized C-fibers, but not mechanosensitive Aδ-fibers, are inhibited by sildenafil. There was no significant difference between female and male mice in these studies.

Bladder cross-section imaging studies (Aim 1c):

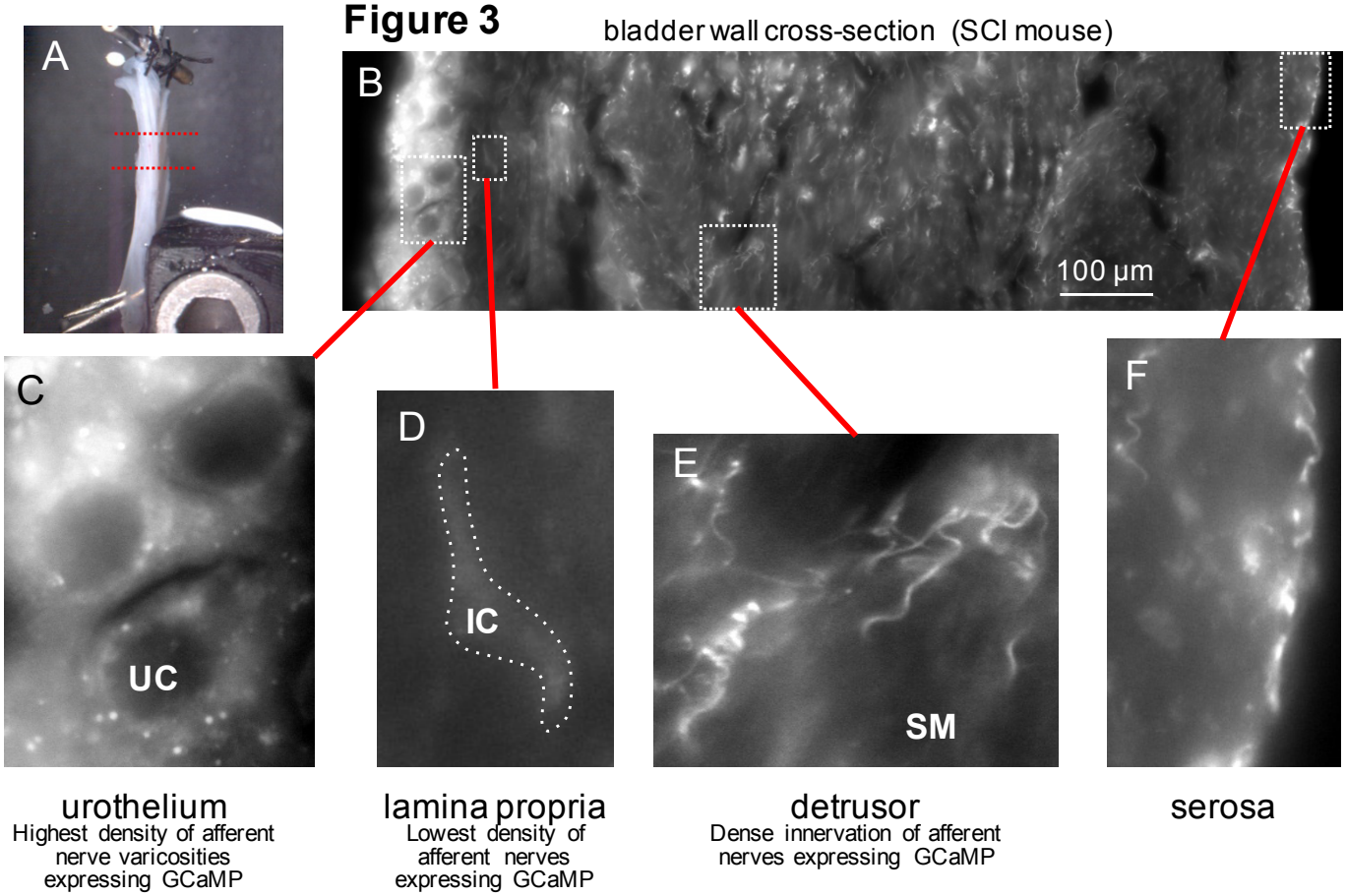
One of the consequences of SCI is extensive neural remodeling at various locations. However, the functional implication of peripheral remodeling within the bladder wall is not fully understood. During the project period a new technique became available that allowed us to selectively label and record Ca^{2+} transients from afferent nerves. The technique originally proposed in the project relied on muscle activity and results were more difficult to interpret. Thus, to study afferent activity within the bladder wall, we performed L6-S1 dorsal root ganglia (DRG) injections with recombinant pseudorabies virus (PRV) expressing a Ca^{2+}-sensitive fluorescent protein (GCaMP, **figure 2A-C**). This allowed bladder afferents to be selectively labeled and functionally assessed (through recording of Ca^{2+} transients) within the innervated tissues (Aim 1b and c). These experiments were performed on 20 control and 20 SCI mice (10 females and 10 males each). Unfortunately, we lost 28 animals while adjusting the virus dose and injection protocol.

Figure 2



Fluorescent imaging of DRG-injected SCI mouse bladders demonstrated there was extensive labeling throughout the bladder wall (**figure 3A and B**). The urothelium showed the highest density of fluorescence (figure 3C) followed by the detrusor layer where individual axons could be distinguished (figure 3E and F). The lamina propria had the lowest level of GCaMP fluorescence (figure 3D), which was surprising, as this region is densely innervated by sensory neurons. This suggests that there is no direct cellular interaction of afferents with interstitial cells. There was no difference between female and male bladders. This unique technique offers the opportunity to examine afferent function within the bladder wall following SCI.

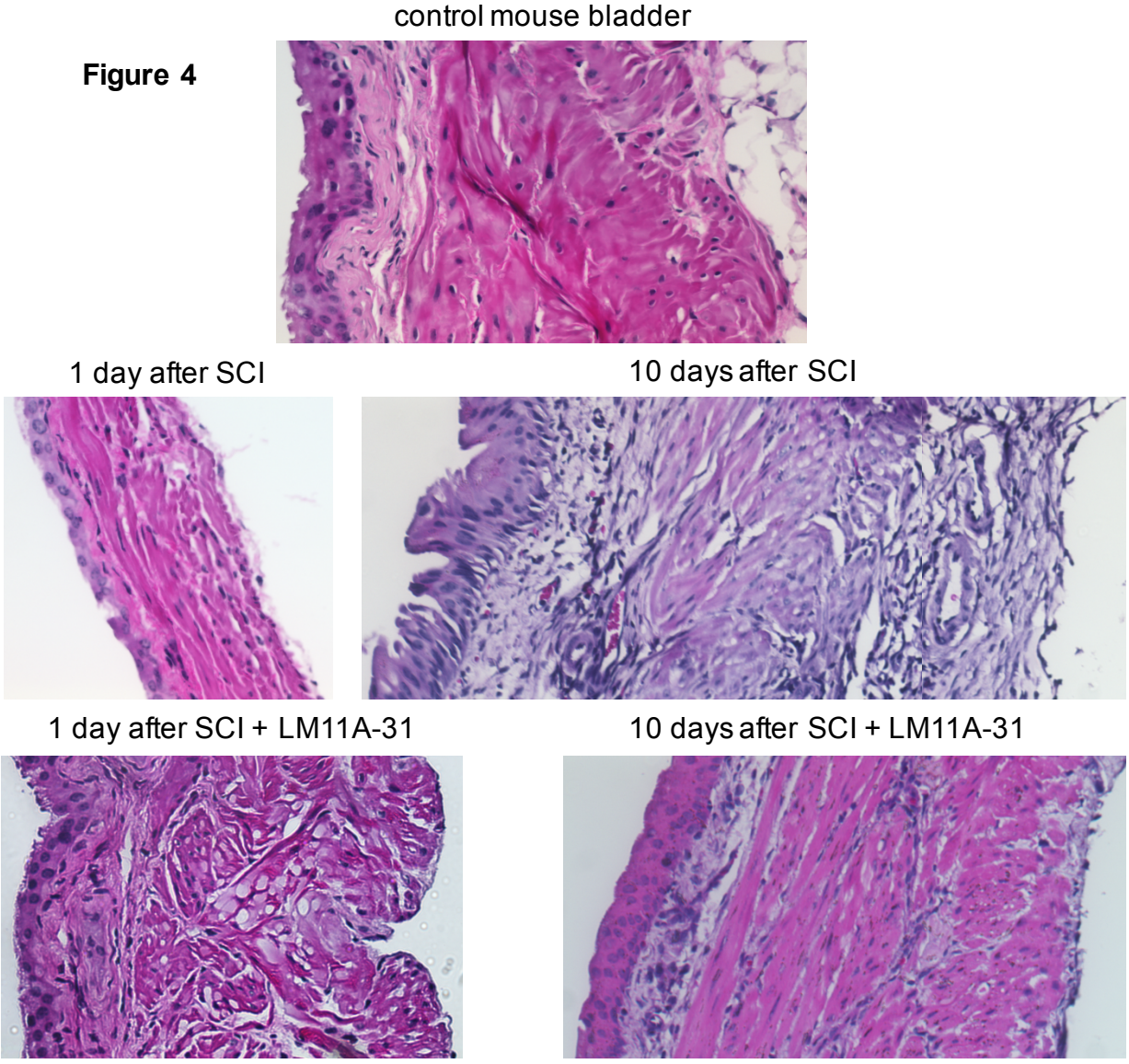
Figure 3



Characterize bladder hypertrophy following SCI (Aim 1a):

p75 is a neurotrophin receptor which preferentially binds uncleaved proneurotrophic factor (proNGF) and pro brain derived neurotrophic factor (proBDNF) to trigger apoptosis, while mature neurotrophins promote cell growth *via* Trk and p75 receptors. Proneurotrophins are produced in the central nervous system under pathologic conditions, including T8-T9 SCI, and regulate cell apoptosis. Since neurotrophins and their receptors are present in all tissues including those of the bladder, we hypothesized that the pro forms may account for observed loss of urothelial cells and barrier function and for development of DSD following SCI. Thus, our study of the role of proneurotrophin binding to p75 receptor in SCI-induced bladder dysfunction relates to both Aims 1 and 2. In SCI, p75 is upregulated in the urothelium and proneurotrophins in the bladder wall (not shown). Bladders from control and SCI mice treated and untreated with LM11A-31, the ligand blocking the binding of proneurotrophins to p75 receptor, 100 mg/kg/day starting one day prior to SCI, were dissected 1 or 10 days following SCI, flattened, fixed in 10% formalin and embedded in paraffin (n = 6 for each group and gender). Embedded tissues were serially cut in 3 µm thick sections, deparaffinized and stained with Hematoxylin and Eosin. Histological analysis of bladders from untreated SCI mice showed a loss of urothelial integrity and detrusor atrophy at day 1 post injury. Urothelial hyperplasia and detrusor hypertrophy were observed at day 10 post-SCI as a consequence of DSD and bladder overdistension (**figure 4**, middle panels). However, in animals treated daily with LM11A-31, the urothelial layer was preserved and the detrusor muscle not hypertrophied (figure 4, lower panels), similar to control.

Figure 4



We have also demonstrated that LM11A-31 improved bladder function using decerebrated cystometry 10 days after SCI. Mice were anesthetized using 1-3% isoflurane followed by a small incision in the neck exposing the carotid arteries and the trachea. Ligatures were placed around the carotids to decrease blood flow to the brain and a tracheotomy performed using PE-60 tubing. The open end of the tube was connected to an anesthesia delivery system, a craniotomy performed and the brain rostral to the supracollicular level sectioned away. Decerebration allowed us to employ cystometry in animals without the use of anesthetics which can block reflex bladder contractions. PE-50 catheter was inserted through a hole in the bladder dome, secured using a suture and connected to a pressure transducer and syringe pump. The bladder was filled with saline at 0.01 ml/min until reflex contractions were elicited to perform voiding cystometry. Following voiding cystometry, the urethra was closed with a suture and isovolumetric studies performed. Again, treated and untreated with LM11A-31, control and SCI mice were used (n = 6 in each group and gender).

In these studies, LM11A-31 administration resulted in greatly improved bladder compliance and voiding function in both genders, as seen in **figure 5** compared to untreated SCI animals. Treated mice exhibited efficient voiding, long intercontractile intervals, high bladder compliances and the absence of detrusor hyperreflexia. To the best of our knowledge, LM11A-31 may be the first therapeutic agent to improve bladder function to this extent following SCI.

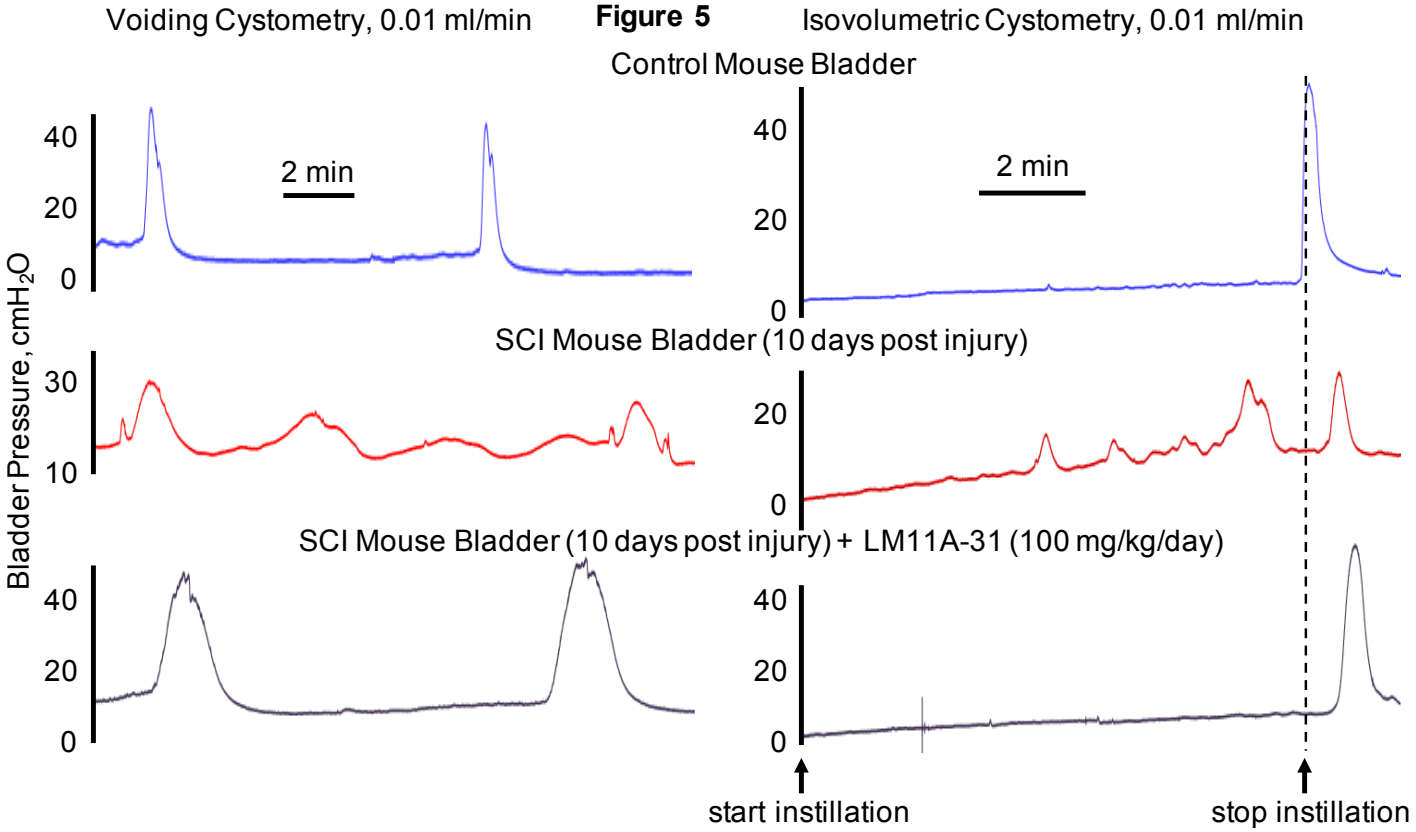


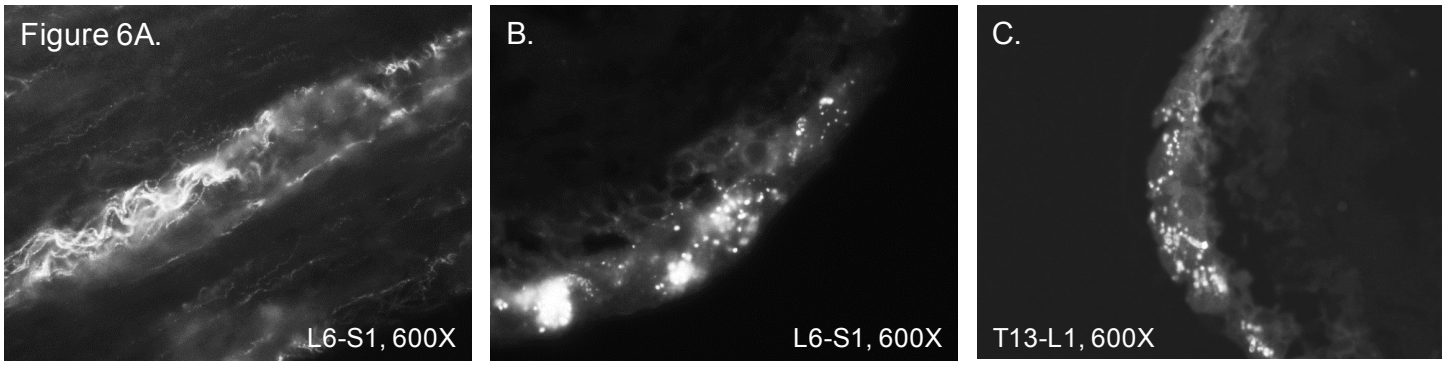
Figure 5

Bladder permeability studies (Aim 2d):

Mice were anesthetized with isoflurane and bladders excised, washed with oxygenated Ringer's solution, mounted on a nylon ring (0.26 cm^2 exposed area) and placed in an Ussing chamber under constant stirring and temperature control. TER as a measure of permeability was determined by passing current through a set of Ag – AgCl electrodes and measuring the resulting voltage deflection (*Vt*) when current is stepped. All data were outputted to a computer for display, analysis and storage. These experiments were done in control and SCI mice as well as in mice treated with LM11A-31 (100 mg/kg/day) 1 day following SCI (n = 6, males and females). We have shown that the bladders from control mice with intact spinal cords exhibit TER of 2,750 ± 1,000 $\Omega \bullet cm^2$. However, 24 hours after SCI, TER was significantly decreased (600 ± 200 $\Omega \bullet cm^2$) demonstrating the disruption of urothelial layer. LM11A-31 treatment preserved the high TER (2,000 ± 400 $\Omega \bullet cm^2$). This demonstrates that LM11A-31 can also be effective in the periphery acting on $p75^{NTR}$ in urothelial cells.

Role of UC in DO (Aim 2):

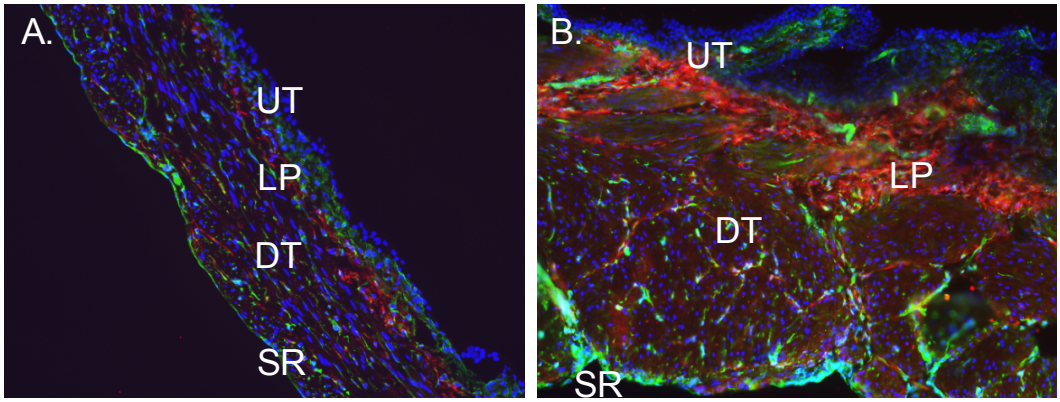
Another important finding from our studies was bidirectional communication between the urothelium and afferent nerves. Fluorescent beads injected in to L6-S1 (pelvic) and T13-L1 (hypogastric) DRG of control mice (n = 10, females and males) showed accumulation in the nerve axons/varicosities and within the urothelial layer (**figure 6**). These experiments were performed to compare the dispersion of fluorescent tracers *versus* viral constructs. The implication from these studies is that there is an intimate connection between the urothelium and sensory nerves, which may be critical for normal bladder function. Further investigation is necessary to determine the role of this mechanism in bladder overactivity following SCI.



Role of IC in DO (Aim 2):

Effects of SCI on IC expression in the bladder wall was studied using immunostaining of tissue sections (Aim 2a). Bladders from control and SCI mice (n = 6 each, males and females) were dissected, flattened, embedded in OCT and frozen sections were serially cut 7 µm thick. **Figure 7** shows examples of normal (7A) and SCI (7B) mouse bladder cross-sections labeled for the IC markers, vimentin (green) and CD34 (red), magnification x100. Following SCI, there was significant remodeling of the bladder wall in both genders. Specifically, there was hypertrophy of the detrusor (DT), and hyperplasia of the urothelium (UT) and IC (especially in the lamina propria, LP). The increase in the number of IC was greater than that of UC, which we propose decreases the inhibitory effects of urothelial-produced NO• on IC, thereby increasing their pacemaker activity which can drive spontaneous contractions and intrinsic detrusor overactivity.

Figure 7



Evaluate new therapies for reducing afferent sensitization and detrusor overactivity in SCI (Aim 3):

Effects of β3-adrenoceptor agonist and BTX-A in SCI mice (Aim 3a and b):

We evaluated the effect of a β3-adrenoceptor agonist (BRL-37344) on urinary bladder function and afferent nerve activity in control and SCI (T8-T9 transection) mice of both genders. We have also assessed the effect of BTX-A as well as BTX-A and β3-adrenoceptor agonist combination therapy. Cystometry and afferent recording methods were described above in Aims 1a and 1b, respectively. Control and SCI mice, untreated and injected with BTX-A, were used in these studies.

Adult female and male C57Bl/10 mice (n = 10 each group and gender) were used for *in vivo* decerebrate cystometry and *in vitro* bladder-pelvic spinal nerve (L6-S1) recordings. For nerve recordings, bladders were connected to a tension transducer and spinal nerves were passed into adjacent oil recording chambers. The bladders were stretched *via* a computer controlled stepper motor to evoke mechanosensitive firing. The β3 agonist (10nM – 1μM) was added to the perfusate. For cystometry, the β3-adrenoceptor agonist or antagonist (L-748,337) were given IP at 0.5 mg/kg. All the studies were done in the presence of 100nM propranolol to block β1- and β2-adrenoceptors. BTX-A was injected (2 units) *in vivo* into mouse bladder walls. After 48 hours, the animals were used for cystometry or their bladders were excised for nerve recordings. When injected IP, 1 unit of BTX-A is, by definition, the LD50 in mice. However, when injected into the bladder wall, 2 units are not lethal but decrease nerve mediated contractions by 70%.

In control mice, addition of β3-adeonceptror agonist did not alter stretch-evoked afferent activity (not shown). However, SCI mice exhibit large amplitude spontaneous detrusor contractions and afferent firing not seen in controls. The spontaneous activity was eliminated by β3-adrenoceptor agonist while stretch-evoked firing was not affected (**figure 8**).

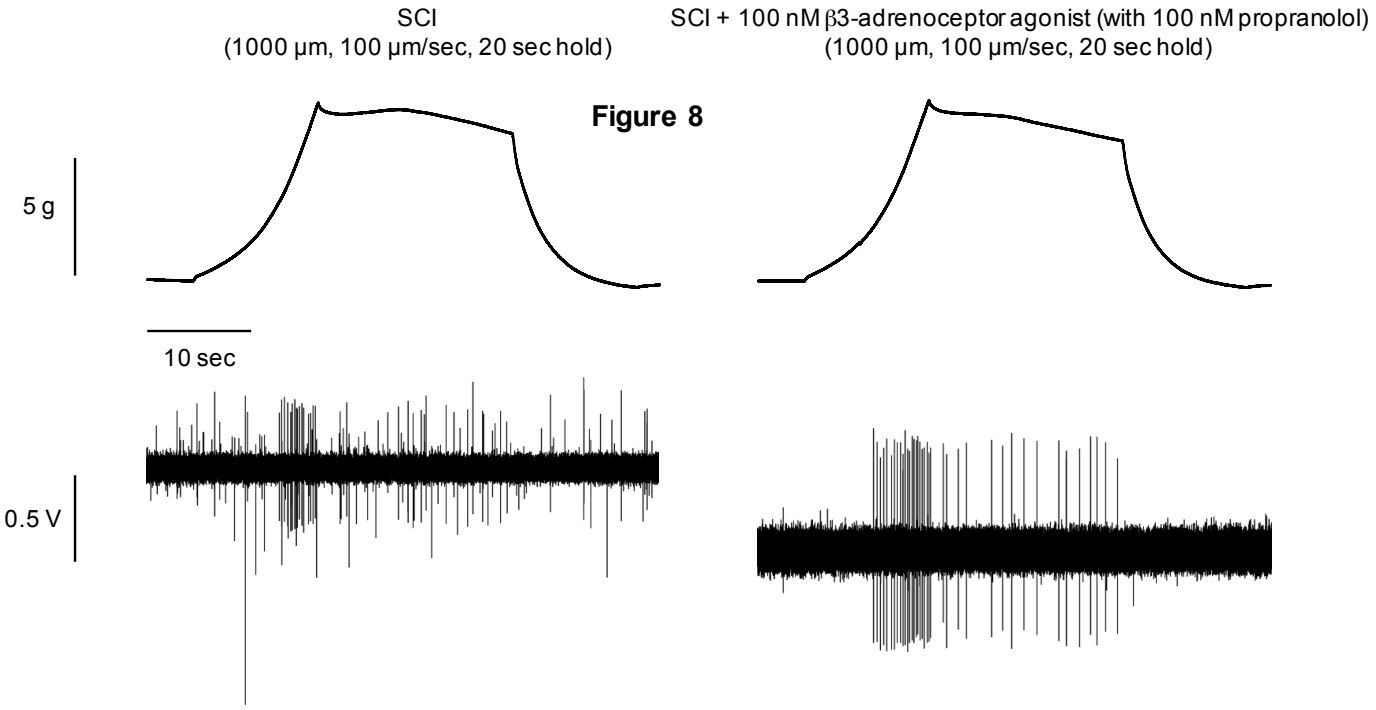


Figure 8

The efficacy of β3-adrenoceptor agonist was enhanced in SCI rodents treated with BTX-A (**Figure 9**).

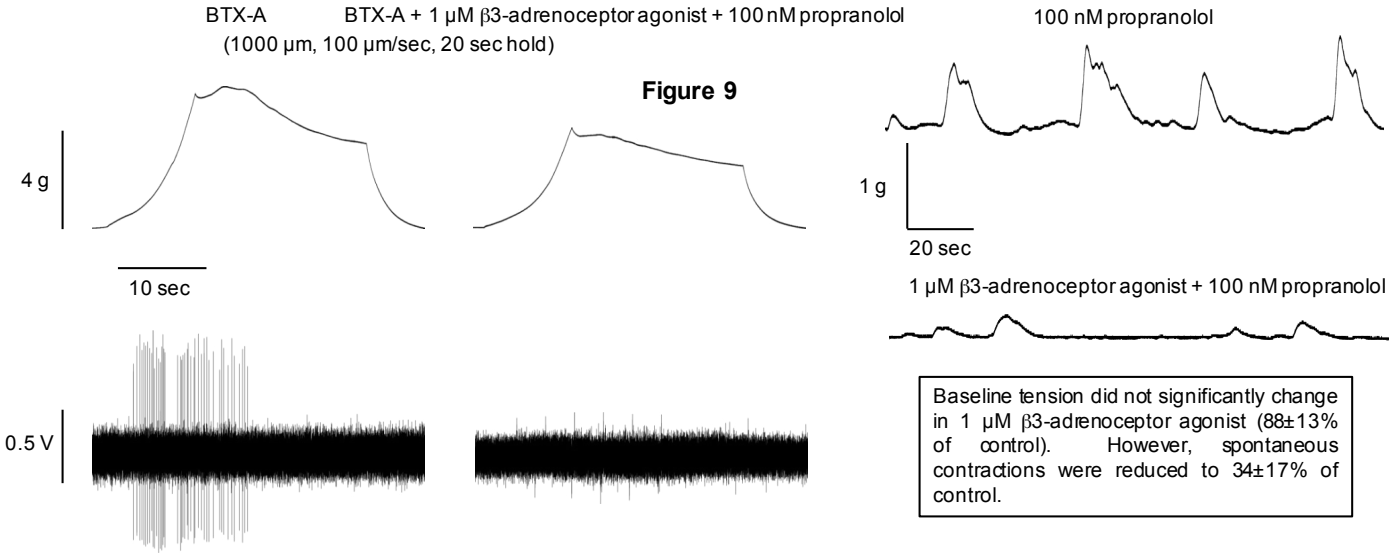


Figure 9

Baseline tension did not significantly change in 1 µM β3-adrenoceptor agonist (88±13% of control). However, spontaneous contractions were reduced to 34±17% of control.

In cystometric studies of the bladders of untreated control mice, the β3-adrenoceptor agonist was without effect (**figure 10A**). However, in SCI mice, β3-adrenoceptor agonist was beneficial by abolishing intrinsic bladder contractions and increasing compliance (figure 10B). In BTX-A treated SCI mice, β3-adrenoceptor agonist dramatically improved bladder compliance which was decreased by the toxin (figure 10C).

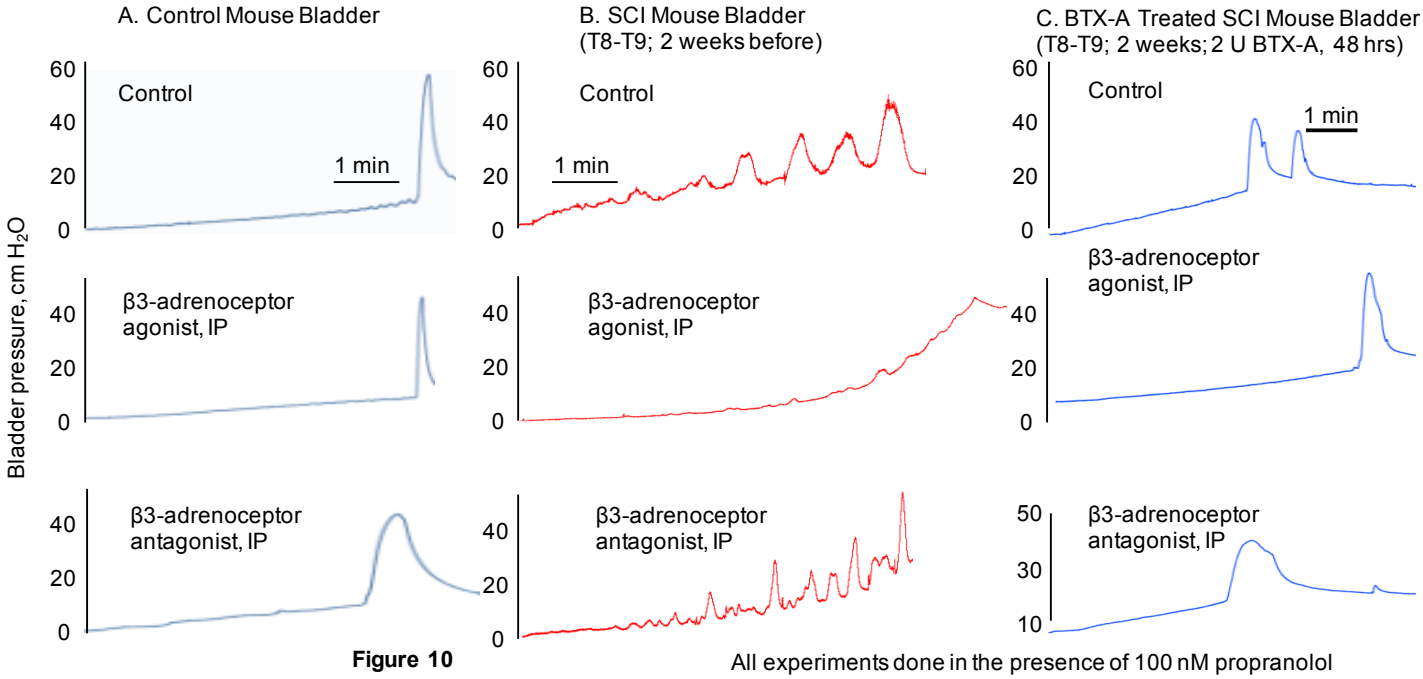


Figure 10

All experiments done in the presence of 100 nM propranolol

The results in figure 10 suggest that β3-adrenoceptors do not have a significant role in normal mouse bladders of either gender and that bladder relaxation in these animals may be mediated by the β2- or β1-adrenoceptor receptor subtype. Following SCI, however, our data suggest that β3 receptors are upregulated. While the β3-adrenoceptor subtype predominates over β1 and β2 in human bladders, it may also be upregulated in pathology thereby increasing the efficacy of β3 agonists. Moreover, in SCI patients treated with BTX-A, combination with a β3-adrenoceptor agonist may be beneficial by improving bladder function.

$P2Y_6$ antagonist studies (Aim 3c):

Although $P2Y_6$ antagonist studies were the part of aim 3 (3c), these data also apply for the aims 2 (2a) and 1 (1c). Expression of $P2Y_6$ receptors was increased following SCI in the urothelium and lamina propria, studied using immunohistochemistry methods in frozen sections (control and SCI bladders, n = 10, females and males). Furthermore, $P2Y_6$ co-localized with CD34-positive IC and not vimentin-positive IC (**figures 11A** and **12**). Bladder sheet tension recordings (for methods see Aim 1b) demonstrate $P2Y_6$ receptor antagonist can reduce spontaneous contractions in SCI but not control mouse bladders, n = 10 (figure 11B).

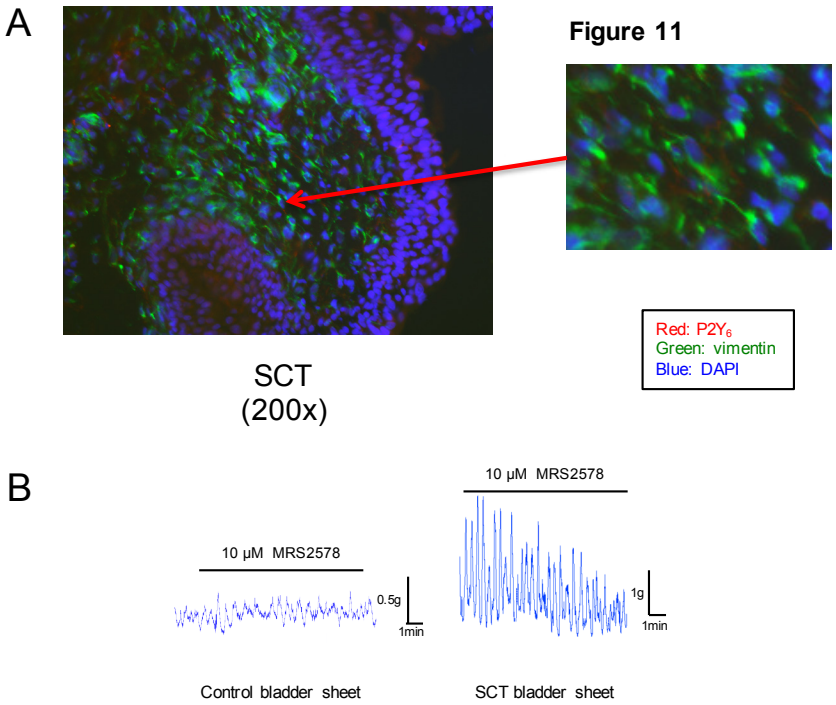
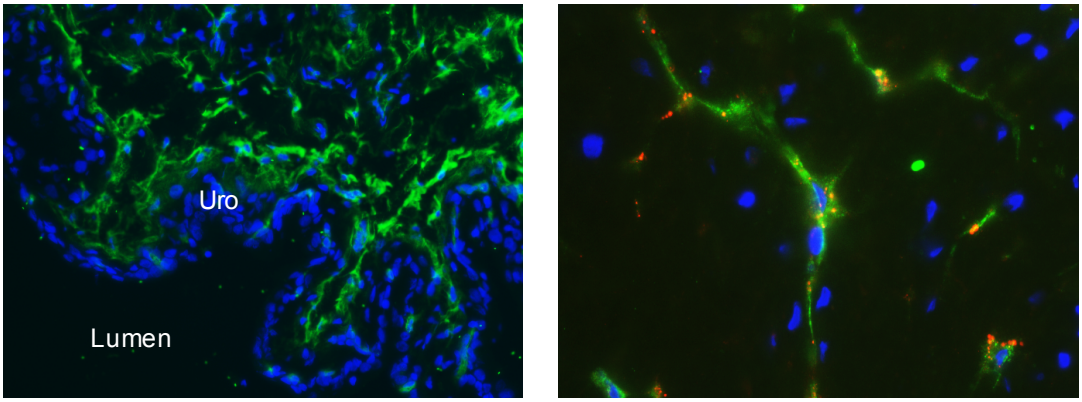


Figure 11



Control (200x)

Green: CD34
Blue: DAPI

Figure 12

SCT (600x)

Red: $P2Y_6$
Green: CD34
Blue: DAPI

This suggests there are different populations of IC within the bladder wall which may have distinctive functional properties. As vimentin + c-kit positive IC have been associated with pacemaker activity, $P2Y_6$ + CD34 IC could potentially be involved in signal transduction from the urothelium to the smooth muscle layer to affect spontaneous contractions.

Chronic tibial nerve stimulation in SCI mice (Aim 3d):

First, we tested chronic TNS for persistent inhibition of enhanced reflex contractions due to bladder irradiation in adult mice. This model was used so that TNS could initially be tested in animals with an intact central nervous system. To induce irradiation cystitis, female and male mouse bladders were withdrawn from the abdomens through a small incision and irradiated using linear irradiator with 10 Gy. Two weeks later, an alligator clip electrode was connected to a mouse right hind limb and stimulation (10 Hz, 2 ms, 15-20 V) was performed for 1 hour / day for 5 days. Two to three days after TNS was completed, decerebration was performed and empty bladders were filled with saline at 0.01 ml/min until reflex contractions were elicited for cystometrograms. Pressure threshold, maximal voiding pressure and the filling time were measured. The difference between data sets were tested with Student's t-test, significance was when $p<0.05$. The decerebration did not abolish the therapeutic action of TNS. Control and irradiated, stimulated and non-stimulated mice were used for these studies (n = 10).

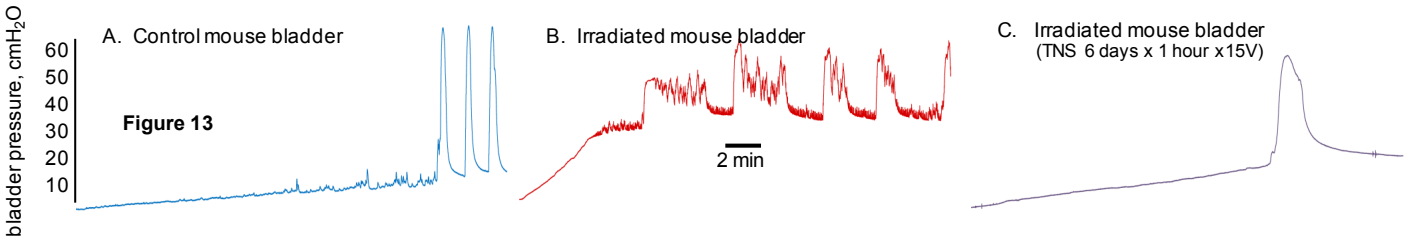
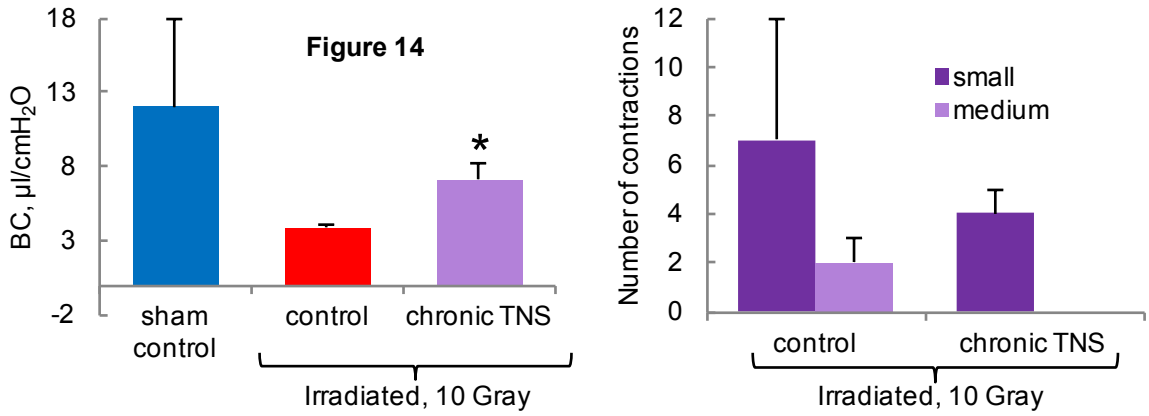


Figure 13

After irradiation, bladders exhibited decreased filling times, increased pressures (**figure 13B**) and decreased bladder compliances (BC) calculated as the saline volume infused to elicit a reflex contraction divided by the pressure threshold (3.6 ± 1.0 µl/cmH_2O *versus* 12.0 ± 6 µl/cmH_2O in controls, **figure 14**).

The number of small (< 5 cmH_20) and medium (5 - 15 cmH_20) contractions during the filling phase increased dramatically (7 ± 5 *versus* 3 ± 2 and 4 ± 1 *versus* 0 ± 0, respectively, figure 14). Cystometries of the bladders treated with chronic TNS demonstrated that this treatment significantly increased bladder compliances (7.1 ± 1.0 µl/cmH_2O, $p<0.01$ *versus* irradiated non-treated mice), decreased developed pressures and the number of small contractions (figure 14). Chronic TNS also eliminated the medium size contractions. These results were observed in both genders.

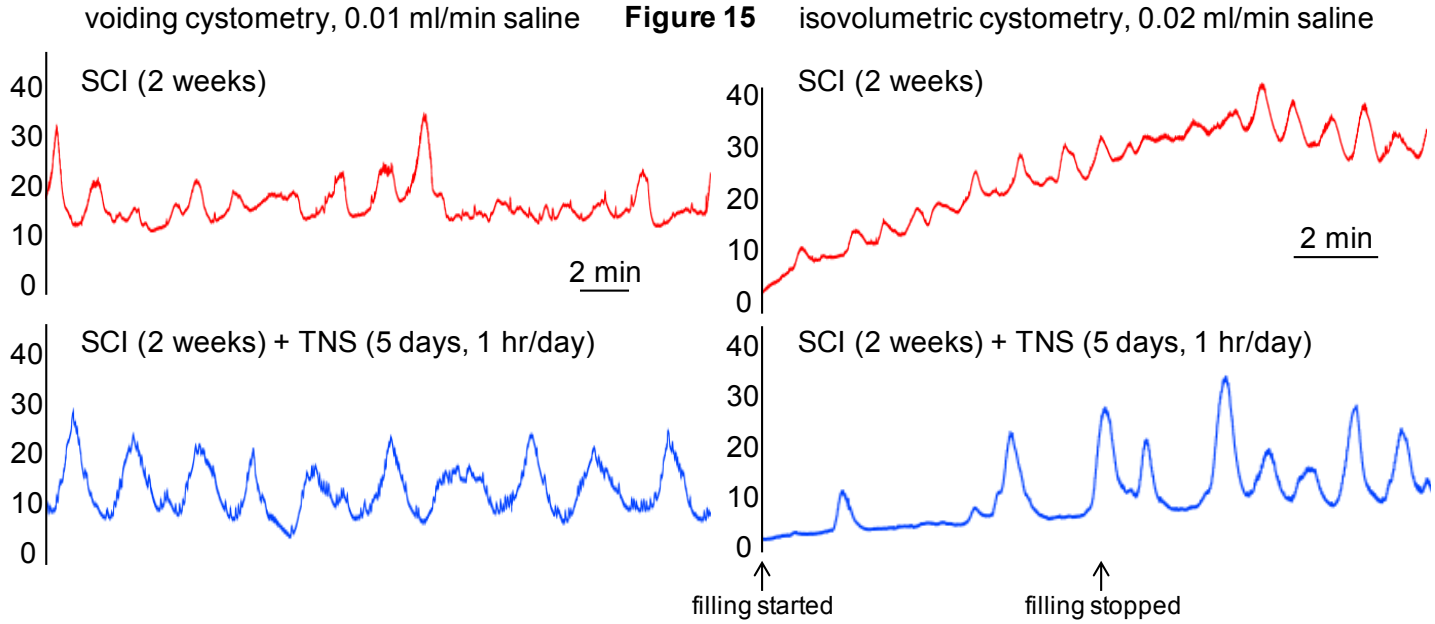


* - p<0.01, *versus* irradiated control.

1 Gray = 100 rad

It was the first animal model demonstrating chronic inhibition of bladder overactivity by TNS.

Subsequently, we tested TNS with the parameters reported to be effective, on mice with spinal cord transection at T8-T9 levels. Again, an alligator clip electrode was connected to a mouse right hind limb and stimulation (10 Hz, 2 ms, 15-20 V) was performed for 1 hour / day / 5 days starting one week after injury. All experiments were carried out on n = 10 female and male mice for control and SCI groups with and without TNS. **Figure 15** and **the table** below demonstrate that TNS did not have a significant effect in SCI animals. SCI bladders treated with TNS were as large as untreated ones (a consequence of DSD) and exhibited non-voiding contractions (figure 15).



We conclude that the site of TNS is supraspinal but subcortical as decerebration did not abolish the effects of TNS on bladder function following irradiation.

	Time to contraction, min	BCP, µl / cm H_2O	BC, µl	Number of small (< 5 cmH_2O) contractions	Number of medium (> 5 cmH_2O) contractions	Bladder weight, mg
control	12 ± 6	12 ± 6	120 ± 50	3 ± 2	0	20 ± 2
irradiated bladder	4 ± 2	3.6 ± 1	40 ± 29	7 ± 5	4 ± 1	20 ± 2
irradiated bladder with TNS	10 ± 2	7.1 ± 1	100 ± 25	0	0	19 ± 3
SCI	9 ± 3	39 ± 7	180 ± 60	4 ± 3	3 ± 2	70 ± 9
SCI+TNS	14 ± 3	41 ± 5	280 ± 60	1 ± 1	3 ± 1	69 ± 7

In total, **748 mice** were used; 360 females, 360 males and 28 unexpected fatalities. 124 mice – for afferent recordings, 288 – for cystometry, 40 (+ 28 unexpected fatalities) and 20 mice for viral and bead injections, respectively, 48 – for TER measurements, 40 – for tension recordings, 64 – for immunochemistry and 96 – for histological studies.

Key Research Accomplishments

- SCI induces lamina propria interstitial cell hyperplasia and coupling enhancing pacemaker function leading to bladder overactivity.
- PDE5 inhibitors increase nitric oxide levels which uncouples interstitial cells leading to decreased bladder overactivity.
- PDE5 inhibitors treat neurogenic bladder overactivity by selectively desensitizing c-fiber afferents.
- SCI increases interaction of proneurotrophins with p75 receptors that leads to both central (DSD) and peripheral (loss of urothelium) pathological effects.
- P75 receptor antagonists are one of the most effective agents in SCI-induced lower urinary tract dysfunction. Oral administration of LM11A-31 prevents loss of the urothelium, bladder hypertrophy and the development of DSD.
- There is no communication of lamina propria interstitial cells with afferent nerves.
- There is bidirectional communication between UC and afferent nerves.
- β3-adrenoceptor agonists treat both neurogenic and myogenic overactivities by selective inhibition of c-fiber afferents and interstitial cells, respectively.
- β3-adrenoceptor agonists inhibit nociceptive but not stretch-sensitive afferent nerves. This should decrease painful nociception without adversely affecting stretch-evoked micturition.
- BTX-A inhibits nociceptive and stretch-sensitive afferent nerves. While this will decrease painful sensation, given that BTX-A also inhibits parasympathic nerves, this may inhibit bladder contraction requiring catheterization.
- β3-adrenoceptors are normally absent in mouse bladders but upregulated following SCI. While these receptors are present in human bladders, they may also be upregulated in pathology making β3-adrenoceptor agonists more efficacious.
- β3-adrenoceptor agonists increase bladder compliance while BTX-A decreases it. Accordingly, in cases where BTX-A is used, combination with a β3-adrenoceptor agonist may be therapeutically beneficial.
- We have accomplished the first prolonged suppression of bladder overactivity using chronic TNS in an animal model. However, we had to use an irradiated mouse bladder preparation to determine that an intact spinal cord is necessary. Using our decerebrate mouse model we have demonstrated that the cortex in not involved in the prolonged effects of TNS neuromodulation. Accordingly, TNS is ineffective in treating SCI-induced bladder overactivity as its site of action is supraspinal but subcortical (*i.e.*, in the brainstem).

Conclusion

Aim1

We developed a methodology to selectively record Ca^{2+} transients from bladder afferent nerves using recombinant PRV expressing fluorescent Ca^{2+} sensors. This methodology has an advantage over the previously proposed optical mapping technique using Ca^{2+} sensitive dyes, as signals from no other cell type is recorded (*i.e.* smooth muscle). This technique will be used for further investigation of the functional interactions between afferent nerves and cells in the bladder wall.

We have demonstrated that PDE5 inhibitors selectively target sensitized afferent nerves without affecting mechanosensitive ones responsible for initiation of a micturition contraction. The potential mechanism of action on afferent terminals may include inhibition of N-type Ca^{2+} channels and/or enhanced activity of K^+ channels *via* neurokinin 1 and 2 (NK_1 and NK_2) receptors. The efficacy of PDE5 inhibitors on dampening afferent firing demonstrates the importance of NO•-mediated inhibition and could represent a mechanism of action for amelioration of bladder sensory symptoms described in men taking PDE5 inhibitors for erectile dysfunction.

Our last study demonstrated the benefits of orally administered p75 neurotrophin receptor antagonist, LM11A-31, in treating SCI-induced lower urinary tract dysfunction. Following SCI, p75 is upregulated in the urothelium, and proNGF/proBDNF in the bladder wall, promoting apoptosis and disruption of the urothelial barrier. Following cleavage, the mature NGF/BDNF selectively bind to TrkA/TrkB receptors, promoting nerve regeneration. This may explain why studies by others using NGF/BDNF antibodies or siRNA knockdown were only marginally effective in treating bladder dysfunction as they inactivate both the pro (beneficial) and mature (harmful) forms.

Aim2

We have demonstrated, using DRG injections with fluorescent beads, that a bidirectional communication mechanism exists between the urothelium and innervating afferent nerves. The functional significance in normal bladder activity and the alterations following SCI is still not fully understood and is one of the focuses for future studies.

SCI resulted in significant structural changes to the bladder wall including urothelial and IC hyperplasia and detrusor hypertrophy. The increase in the number of IC was significantly greater than that of the urothelium which could result in insufficient urothelial NO• which can result in i) increased coupling of the IC network responsible for coordinating spontaneous bladder contractions and ii) afferent sensitization (as demonstrated in aim 1).

Aim3

We assessed the effects of BTX-A and β3-adrenoceptor agonists on bladder function in control and SCI mice to determine if these drugs or their combination were therapeutically beneficial. In SCI mice, BTX-A at its lowest effective dose inhibited c-fiber afferent excitability. It is hypothesized that this involves inhibition of neuropeptide release and disinhibition of K^+ channels resulting in hyperpolarization and desensitization of the nerve terminals. β3-adrenoceptor agonists were beneficial and abolished neurogenic overactivity (through selective inhibition of nociceptive afferents), myogenic (intrinsic) bladder contractions and improved bladder compliance. Conversely, in the bladders of control mice the β3-adrenoceptor agonists were without effects because β2-adrenoreceptors mediate relaxation in these animals. These results demonstrate that β3-adrenoceptors are upregulated in mice after SCI. In humans, they are normally present in the bladder wall. These findings suggest that BTX-A and β3-adrenoceptor agonist combination therapy may be beneficial in improving bladder function in SCI patients.

$P2Y_6$ receptors expression increased specifically in the urothelium and CD34-positive IC following SCI. Additionally, $P2Y_6$ antagonist reduced the amplitude of spontaneous contractions in SCI mouse bladders but not controls. These data suggest in SCI, $P2Y_6$ receptors may be involved in signal transduction from urothelial cells to the smooth muscle *via* CD34-positive IC network.

We developed the first animal model demonstrating that chronic TNS (1 hour / day / 5 days) causes persistent inhibition of bladder overactivity in non-SCI mice. We described the development of this animal model where we used irradiation-induced bladder overactivity in order to work with an intact spinal cord and decerebration to remove the CNS. The irradiated and SCI models allowed us to determine that the site of action of TNS is supraspinal but subcortical (*i.e.*, located in the brainstem) and therefore TNS is ineffective in T8-T9 transected mice.

Inventions, Patents and Licenses

P75 receptors antagonist have considerable potential in treating SCI patients. However, we are interested in sharing our findings/discoveries with the scientific and pharmaceutical communities and not in patenting or licensing them.

Reportable Outcomes

Based on findings from this study we are now in the process of synthesizing p75 receptor antagonists that are quaternary compounds and do not cross the blood brain barrier. In this way we will be able to determine if the site(s) of action of these agents are central and/or peripheral.

Publications, Abstracts and Presentations

Abstracts and poster presentations at the Military Health System Research Symposium (MHSRS), 2012:

1. Y Ikeda, I Zabbarova, A Kanai. Use of PDE5 Inhibitors to Treat Bladder Overactivity due to Spinal Cord Injury.
2. I Zabbarova, L Birder, W de Groat, Y Ikeda, A Wolf-Johnston, A Kanai. Use of Chronic Tibial Nerve Stimulation to Treat Bladder Overactivity.

Presentations at the International Consultation on Incontinence – Research Society (ICI-RS) meetings, 2013-2014:

1. A Kanai, Y Ikeda, A Hanna-Mitchel. Do we understand any more about LUT interstitial cells?
2. I Zabbarova, A Kanai, J Gajewski.. Does our limited knowledge of the mechanisms of neuromodulation limit its benefits for patients?
3. A Kanai, Y Ikeda, C Fry. What are the implications for bidirectional communication between afferent nerves and urothelial cells?

Abstracts and poster presentations at the American Urological Association Annual Meeting, 2014

1. Y Ikeda, I Zabbarova, L Birder, W de Groat, A Kanai. Bidirectional communication between afferent neurons and urothelial cells in the mouse urinary bladder.
2. Y Ikeda, I Zabbarova, L Birder, C Korstanje, A Kanai. β3-adrenoceptor agonists selectively inhibit nociceptive bladder afferents.

Abstracts and poster presentations at the Society for Neuroscience Annual Meeting, 2014

1. I Zabbarova, S Yoon, W de Groat, L Birder, A Kanai. p75 Receptor inhibition decreases spinal cord injury-induced bladder damage.
2. Y Ikeda, I Zabbarova, W de Groat, L Birder, A Kanai. Viral probes injected into the mouse tail are expressed in dorsal root ganglia and bladder urothelium; a possible mechanism for the development of interstitial cystitis.

Manuscripts:

1. Y Ikeda, I Zabbarova, L Birder, W de Groat, C McCarthy, A Hanna-Mitchell, A Kanai. Botulinum neurotoxin serotype A suppresses neurotransmitter release from afferent as well as efferent nerves in the urinary bladder. *Eur. Urol.* 62:1157-1164, 2012.
2. A Hanna-Mitchell, A Wolf-Johnston, S Barrick, A Kanai, M Chancellor, W de Groat, L Birder. Effect of botulinum toxin A on urothelial-release of ATP and expression of SNARE targets within the urothelium. *Neurourol Urodyn*, 34:79-84, 2013.
3. A Kanai, C Fry, A Hanna-Mitchell, L Birder, I Zabbarova, D Bijos, Y Ikeda. Do we understand any more about bladder interstitial cells? ICI-RS 2013. *Neurourol Urodyn*. 33:573-576, 2014.
4. J Gajewski, A Kanai, L Cardozo, Y Ikeda, I Zabbarova. Does our limited knowledge of the mechanisms of neural stimulation limit its benefits for patients with overactive bladder? ICI-RS 2013. *Neurourol Urodyn*, 33:618-621, 2014.
5. L Birder, KE Andersson, A Kanai, A Hanna-Mitchell, C Fry. Urothelial mucosal signaling and the overactive bladder. ICI-RS 2013. *Neurourol Urodyn*, 33:597-601, 2014.

Other Achievements

Nothing to report.

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Appendices

1. Y Ikeda, I Zabbarova, L Birder, W de Groat, C McCarthy, A Hanna-Mitchell, A Kanai. Botulinum neurotoxin serotype A suppresses neurotransmitter release from afferent as well as efferent nerves in the urinary bladder. Eur. Urol. 62:1157-1164, 2012.

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3. A Kanai, C Fry, A Hanna-Mitchell, L Birder, I Zabbarova, D Bijos, Y Ikeda. Do we understand any more about bladder interstitial cells? ICI-RS 2013. Neurourol Urodyn. 33:573-576, 2014.

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9. Y Ikeda, I Zabbarova, W de Groat, L Birder, A Kanai. Viral probes injected into the mouse tail are expressed in dorsal root ganglia and bladder urothelium; a possible mechanism for the development of interstitial cystitis, Neuroscience 2014 abstract.

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Botulinum Neurotoxin Serotype A Suppresses Neurotransmitter Release from Afferent as Well as Efferent Nerves in the Urinary Bladder

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Abstract

Background: Botulinum neurotoxin A (BoNTA), which alleviates overactive bladder symptoms, is thought to act predominantly via the inhibition of transmitter release from parasympathetic nerves. However, actions at other sites such as afferent nerve terminals are possible.
Objective: To evaluate the effects of BoNTA on bladder afferent neuropeptide release and firing.
Design, setting, and participants: One side of the bladder of control and chronic (1–2 wk) spinal cord transected (SCT; T_8-T_9) adult female mice was injected with BoNTA (0.5 U/5 μl saline). After 48 h, bladders with L_6-S_2 spinal nerves were prepared for in vitro recordings.
Outcome measurements and statistical analysis: In bladder preparations, tension and optical mapping of Ca^{2+} transients were used to measure intrinsic contractions, those evoked by capsaicin or the electrical stimulation of spinal nerves. Afferent firing was evoked by stretch or intrinsic bladder contractions. The numbers of responding units and firing rates were measured. Animal numbers were used to detect moderate to large between-group differences based on Cohen's criteria. Two-way analysis of variance was used to test spatial/temporal differences in Ca^{2+} signals as mean plus or minus standard deviation. Differences between data sets were tested with the student *t* test and skewed data sets with a Mann-Whitney *U* test (significant when $p < 0.05$).
Results and limitations: In control and SCT bladders, BoNTA treatment decreased the contractions evoked by electrical stimulation of spinal nerves without altering intrinsic contractions. Afferent firing on untreated sides in response to stretch/intrinsic contractions was increased in SCTs versus controls. On BoNTA-treated sides, afferent firing rates were greatly attenuated in response to mechanical stimulation as were the capsaicin-evoked optical signals mediated by neuropeptide release.
Conclusions: SCT caused an increased sensitivity of afferent nerves to mechanical stimulation that was reduced by BoNTA treatment. Increased intrinsic activity after SCT was unaffected by the toxin. Thus BoNTA suppresses neurogenic detrusor overactivity by targeting afferent as well as efferent pathways in the bladder.

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1. Introduction

Botulinum neurotoxin is produced by *Clostridium botulinum*, and there are seven known serotypes [1]. These toxins target cholinergic nerve terminals by binding to synaptic vesicle protein type 2 (SV2) [2] and inhibiting acetylcholine release through the cleavage of synaptosome-associated protein of 25 kDa (SNAP-25) or vesical-associated membrane protein (Fig. 1) [3,4]. The longest acting serotype is botulinum toxin type A (BoNTA), which has been used clinically for various muscle spasticity disorders. BoNTA has also shown promise in treating neurogenic bladder overactivity [5] by reducing nonvoiding contractions, presumably inhibiting cholinergic efferent activity. Similar effects have been obtained in animal studies. Intravesical BoNTA reduced nonvoiding contractions and increased intercontractile intervals in spinal cord transected (SCT) rat bladders [6]. In addition, BoNTA decreased adenosine triphosphate (ATP) release from the bladder and spinal cord [7].

The ideal outcome for patients with overactive bladder treated with BoNTA injections is reduced urgency and involuntary bladder contractions. However, a potential side effect is urinary retention due to the excessive inhibition of transmission at the parasympathetic neuroeffector junction. Thus BoNTA was initially used in patients with neurogenic disorders such as spinal cord injury, where they overcome retention through catheterization [8]. BoNTA treatment has also been examined in patients with idiopathic bladder overactivity [9] and those with interstitial cystitis/painful bladder syndrome where there were mixed results [10].

Although BoNTA acts on efferent nerve terminals, there may be other sites of action including afferent nerves and the

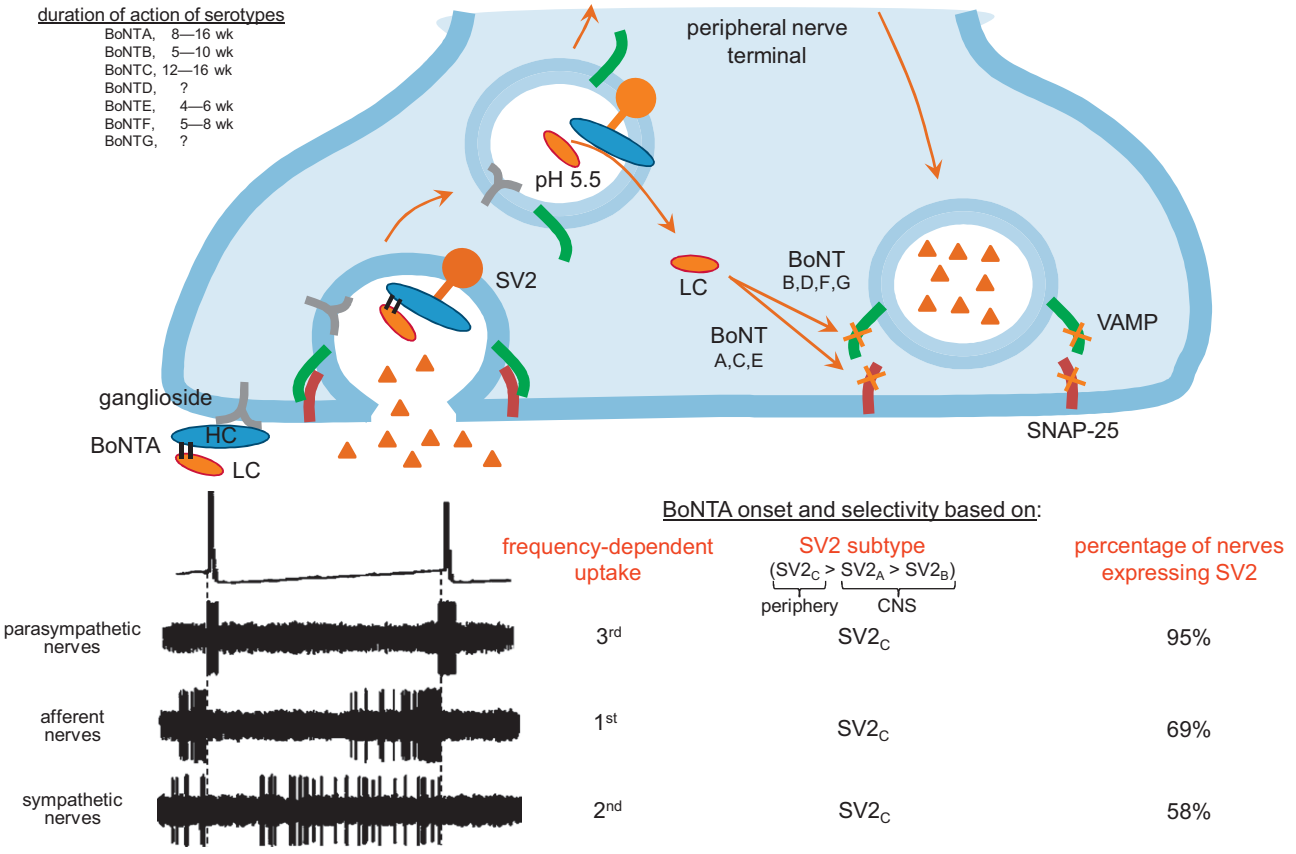

Fig. 1 – Mechanism of action of botulinum toxin type A (BoNTA) and its selectivity for different nerve terminals. Botulinum neurotoxins (BoNTs) initially bind to gangliosides expressed on the outer plasma membrane of nerve terminals [3]. During neurotransmitter release, the interior membrane of the vesicle is exposed, allowing BoNTs to attach to synaptic vesicle protein type 2 (SV2) and be transported into the nerve terminal via endocytosis. The low pH in the endosome causes dissociation of the di-chain polypeptide into its heavy chain (HC) and catalytic light chain (LC) components. The HC then inserts into the vesicle membrane allowing translocation of the LC into the cytosol where it is free to interact with and cleave SNARE proteins. The proteolytic activity and specificity of the LC differs among BoNT subtypes, targeting either synaptosomal-associated protein of 25 kDa (SNAP-25; serotypes A, C, and E) or vesical-associated membrane protein (VAMP; serotypes B, D, F, and G) of the synaptosomal complex.
The uptake of BoNTA depends on the frequency of nerve firing and the percentage of terminals expressing SV2. Although parasympathetic nerves are active only during bladder contraction, they have the highest percentage of nerves expressing SV2 [4]. Sympathetic neurons are active during most of the filling phase but have fewer nerves expressing SV2; this is preferable because their inhibition would decrease noradrenaline release and adversely affect bladder compliance. Afferent nerves progressively become more active as the bladder fills. They have the second least percentage of fibers expressing SV2, but their frequency of firing increases with sensitization, so they may have the highest likelihood of BoNTA uptake in pathology. CNS = central nervous system.

urothelium. A study by a coauthor, using reverse transcriptase polymerase chain reaction and Western blots, revealed SNAP-25 expression in human and mouse urothelium and BoNTA suppression of ATP release from urothelial cells. These data provide further support for the view that the urothelium has "neuronal-like" properties and may participate in bladder sensory mechanisms by releasing chemicals that modulate afferent nerve excitability [10]. However, another laboratory failed to detect SNAP-25 staining in human and rodent urothelium [11].

BoNTA may affect sensory nerves in the bladder through the inhibition of neuropeptide release [12,13] that can prevent autocrine stimulation of neurokinin 1 and 2 (NK1 and NK2) receptors on afferent terminals resulting in decreased afferent firing and sensitization [14]. Increased activity of afferents due to sensitization and bladder stretch may induce a greater uptake of BoNTA (Fig. 1) into afferent rather than efferent terminals that are only active during micturition. BoNTA may also elicit therapeutic effects by decreasing afferent sensitization and sprouting through alteration in afferent terminal receptor expression [15–17], inhibition of nerve growth factor [18], or inflammatory mediator [19] release.

Therefore, BoNTA may affect afferent and urothelial function directly and indirectly. In this study, we examined the effect of BoNTA injections on mechanosensitive afferent nerves in normal and SCT mouse bladders to determine if there is a direct effect that decreases antidromic neuropeptide release and/or firing rates.

2. Material and methods

2.1. Spinal cord transection

The T_8-T_9 SCT surgery was performed as previously described [20]. Animals were expressed by gentle abdominal compression for 1 wk following surgery and used for experiments 1–2 wk after surgery.

2.2. Botulinum toxin type A injections

Mice were anesthetized with isoflurane, and a lower midline abdominal incision was made to expose the bladder. BoNTA (Botox, Allergan Inc; 100-U vials) was reconstituted in 1 ml of sterile saline with a comparable weight (150 kDa) blue dextran dye (0.5 U of BoNTA in 5 μl saline/dextran; Fig. 2A) to track its distribution. Injections were made into the right half of the bladder wall at two to four serosal sites using a 32-gauge needle. The incision was sutured; animals were allowed to recover for 48 h. Based on 300 U/bladder for a 75-kg human, an equivalent dose for a 25-g mouse, which is 3000 times smaller, is 300 U/3000 = 0.1 U. Although 1 U is the median lethal dose for mice when injected intraperitoneally, our studies have demonstrated that it is safe to inject up to 2 U into the bladder wall.

2.3. In vitro afferent nerve recordings

Bladder L_6-S_2 spinal nerve preparations were isolated as previously described [20]. The preparation was mounted in a recording chamber

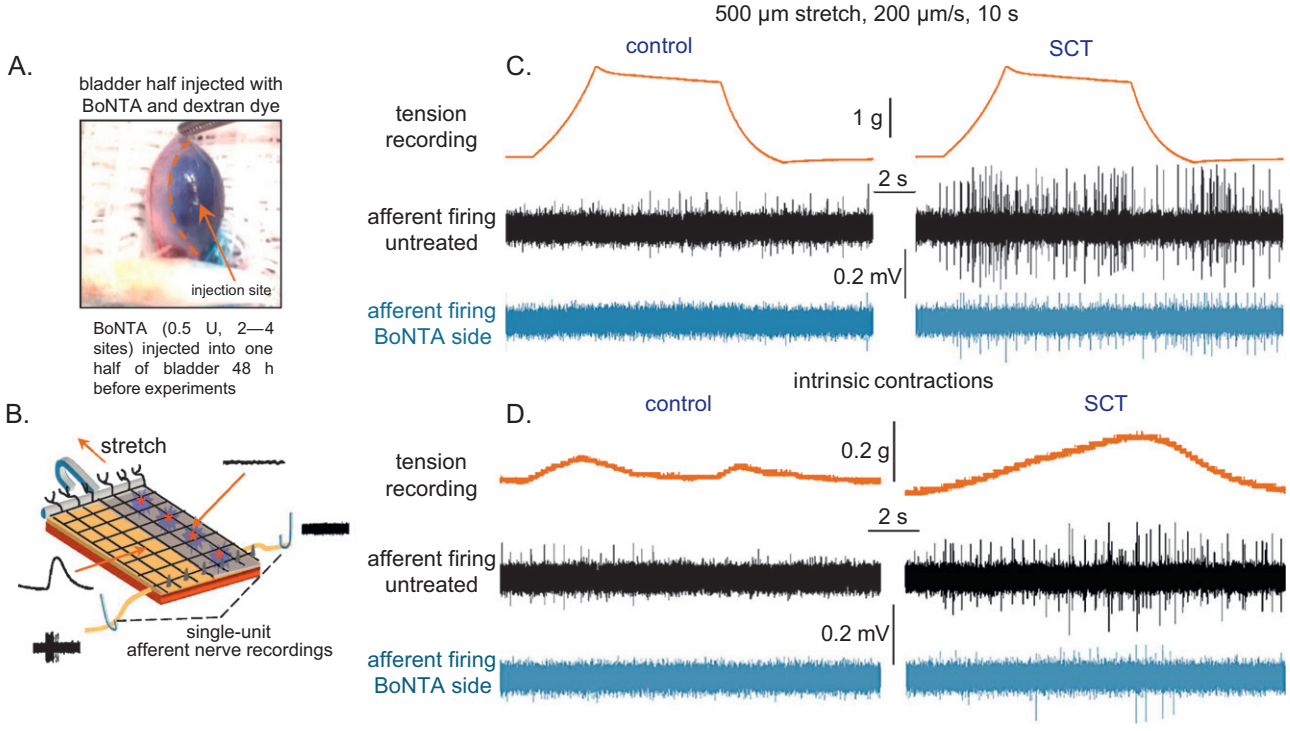

Fig. 2 – In vitro afferent nerve recordings from botulinum toxin type A (BoNTA)–treated mouse bladders. (A) Photo image of a mouse bladder injected across half its surface with 0.5 U of BoNTA in 5 μl of saline and blue dextran dye. (B) Schematic of the bladder-nerve preparation used for recording afferent nerve firing and optical mapping of intracellular Ca^{2+} transients. (C) Simultaneous afferent recordings from BoNTA-treated (blue traces) and nontreated (black traces) sides of the bladder in response to stepper motor-controlled stretches. In normal bladders, stretching resulted in moderate amounts of afferent firing from the control side but not the BoNTA-treated side. In spinal cord transected (SCT) mouse bladders, there was a significantly higher frequency of firing in response to the same stretch protocol; BoNTA inhibited stretch-evoked firing. (D) Intrinsic contractions evoked low-level afferent firing in control bladders. In SCT mouse bladders, the amplitude of intrinsic contractions was greater and resulted in increased frequency of firing compared with controls. BoNTA inhibited afferent firing due to intrinsic contractions in both control and SCT preparations.

(Fig. 2B and 3A), and nerves were passed through gates into oil-filled recording compartments. The bladder was superfused with oxygenated Krebs solution and maintained at 37 °C. For efferent nerve stimulation studies, the spinal nerves were positioned on bipolar platinum electrodes. Nerves were stimulated with a 3-s train at 20 Hz, 0.5-ms pulse width, and 20-V output. The same parameters were used for electric field stimulation studies with bladder cross sections. These parameters were determined to preferentially depolarize neurons and minimize the direct activation of the detrusor when tested in the absence and presence of 1 μM tetrodotoxin (not shown).

For afferent recordings, the spinal nerves were split into smaller bundles and the receptive fields of mechanosensitive fibers located by passing a soft brush along the length of the bladder and then by probing using a von Frey hair. Electrical signals were sampled at 20 kHz, amplified and filtered using a WPI DAM 80 AC differential amplifier interfaced with an AD Instruments PowerLab 8/30. Data were acquired and stored on a computer for offline analysis using LabChart v.7. Electrical signals were fed to an audio monitor to facilitate real-time detection and discrimination of active units.

2.4. Optical imaging of bladder preparations

Bladder sheets were incubated with the Ca^{2+}-sensitive fluorescent dye rhod-2-AM (1 μM) at physiologic temperature for 5 min and imaged using an optical mapping system described previously [21].

2.5. Statistical analysis

Quantitative data are shown as mean plus or minus standard deviation. Differences between normal data sets were tested with the student *t* test; the null hypothesis was rejected when p was < 0.05.

3. Results

3.1. Effect of botulinum toxin type A on mechanically stimulated afferent firing in control versus spinal cord transected mouse bladders

Afferent firing was simultaneously recorded from BoNTA-treated and -untreated sides of the bladders from 10 control and 9 SCT mice in response to controlled stretches (Fig. 2C) and intrinsic bladder contractions (Fig. 2D). Bladders were stretched 500 μm at 10 μm/s and held for 30 s before being relaxed to starting length. On the untreated sides of control bladders, in response to stretch, the firing of a total of 59 units was recorded from six responsive nerves at an average firing rate of 5 ± 0.4 Hz. The intrinsic contraction induced the firing of 48 units at the same rate. BoNTA-treated sides of control bladders were unresponsive to mechanical stimulation. On untreated sides of SCT mouse bladders, both stretch and contraction induced considerably higher activity (stretch: 132 units firing at an average rate of 16 ± 1.8 Hz; contraction: 109 units firing at an average rate of 22 ± 2.3 Hz from six responsive nerves). However, afferent activity on the BoNTA-treated sides of the SCT bladders was significantly decreased ($p < 0.01$): Stretch induced the firing of 42 units at an average of 5 ± 1 Hz, and contraction induced firing at an average of 2 ± 1 Hz in 35 units from four responsive nerves. Control and SCT mouse bladders injected only with dye showed no significant difference in afferent firing compared with nontreated halves (not shown).

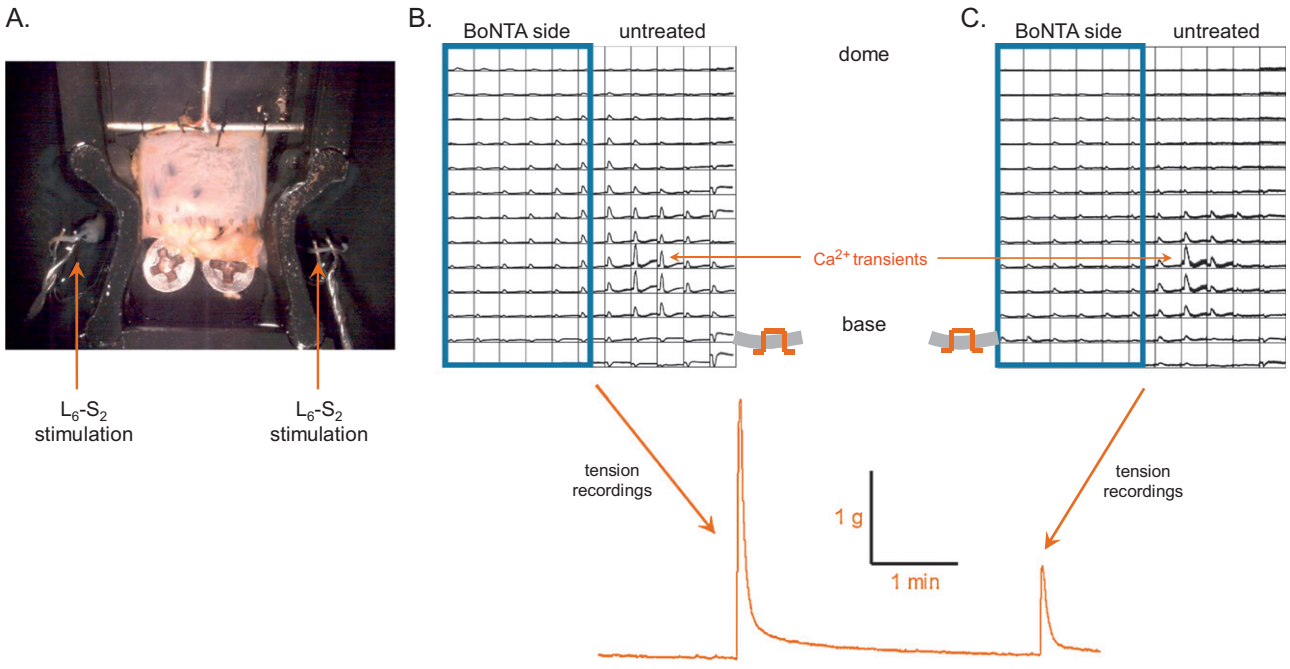

Fig. 3 – Effect of botulinum toxin type A (BoNTA) on nerve-mediated bladder contractions. (A) Photo image of a bladder-nerve preparation with associated L_6-S_2 spinal roots draped over stimulation electrodes. (B) Ca^{2+} optical map from the mucosal surface of a control mouse bladder treated with BoNTA (blue boxed area). Stimulation of nerve roots on the untreated side resulted in a large contraction with the spread of Ca^{2+} activity across the bladder. (C) Nerve stimulation on the BoNTA-reated side resulted in a much smaller contraction and propagation only on the untreated side due to activation of crossed efferent pathways.

3.2. Effects of botulinum toxin type A treatment on efferent pathways in spinal cord transected and normal mouse bladders

Efferent activity was evaluated during electrical stimulation of the left or right spinal nerves, and muscle activity was monitored using Ca^{2+} optical mapping from the mucosal surface along with tension measurements from the sheet. In normal mouse bladders ($n = 11$), nerve stimulation on the untreated side elicited Ca^{2+} transients and tension in the range of 3.03 ± 0.07 g. In contrast, on the BoNTA-treated side, Ca^{2+} transients were essentially abolished, and tension decreased to 1.51 ± 0.56 g ($p < 0.05$) compared with the untreated side (Fig. 3B and 3C). Tension generated when stimulating the spinal nerve on the BoNTA-treated side of the bladder was due to the activation of crossed efferent pathways that project to the untreated side. Similar results were obtained from SCT mouse bladders ($n = 7$). When a bladder cross section was imaged during electrical stimulation, spread of Ca^{2+} activity was not observed in the BoNTA-treated half of the bladder (Fig. 4; $n = 4$ control and 3 SCT bladders). Ca^{2+} activity was suppressed through all the layers of the BoNTA-treated tissue.

3.3. Effect of botulinum toxin type A treatment on antidromic neuropeptide release and intrinsic bladder contractions

Capsaicin (1 μM) was applied to evoke neuropeptide release from transient receptor potential vanilloid (TRPV) 1–containing afferent nerves while mapping Ca^{2+} transients in the muscle that functioned as a sensor (Fig. 5A). Bladders from control mice ($n = 9$) were used to avoid the complication of neuropeptide release due to large amplitude intrinsic contractions. On the untreated side, Ca^{2+} transients were detected mostly near the base/trigone region where most afferent nerves enter the bladder wall. On the BoNTA-treated side, this activity was absent. A second application of capsaicin 15 min later did not elicit a response (not shown), presumably due to the depletion of transmitter and/or the desensitization of TRPV1 receptors, demonstrating that the muscle signals were due to neuropeptides released from afferent terminals.

Following SCT, there was a significant increase in the amplitude of intrinsic bladder contractions compared with normal bladders (peak amplitude from baseline tension: 0.10 ± 0.04 g control vs 0.55 ± 0.21 g SCT; $n \geq 6$; $p = 0.05$). BoNTA treatment did not reduce the amplitude or frequency of these contractions compared with untreated SCT bladders (not shown). The mapping of intracellular Ca^{2+} transients responsible for intrinsic contractions also demonstrated that these are not affected by BoNTA (Fig. 5B). This is supported by previous findings where we showed that intrinsic activity is not blocked by tetrodotoxin and therefore not mediated by action potentials in nerves [21].

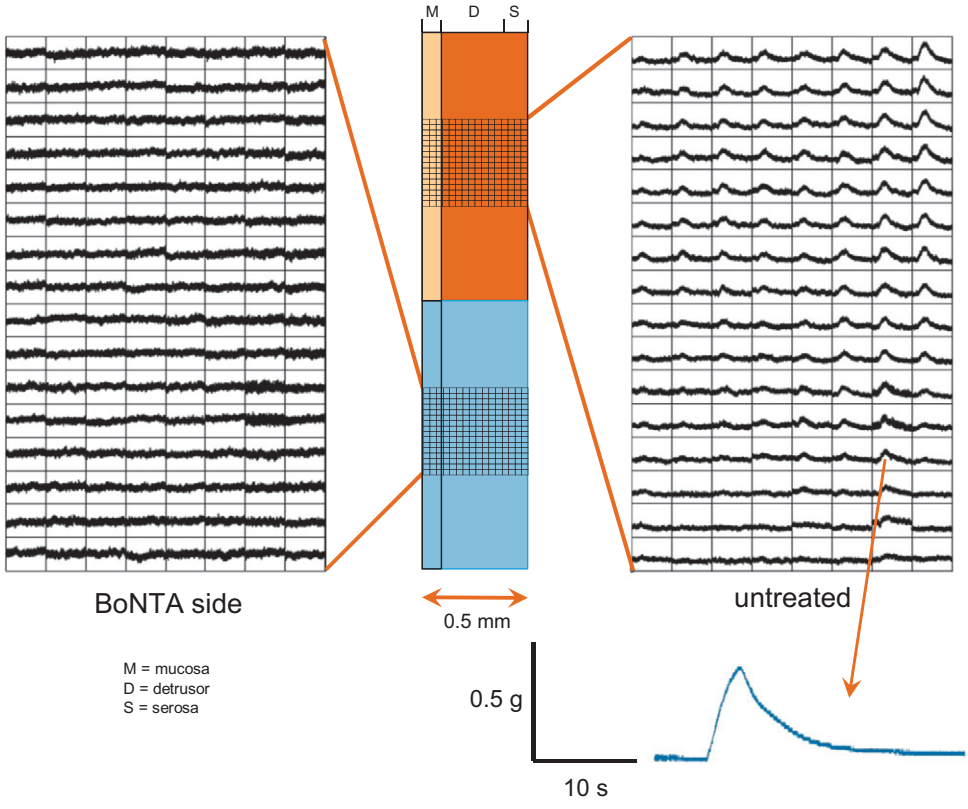

Fig. 4 – Ca^{2+} optical maps from wall cross-sections of normal mouse bladders. The bladder was imaged across the wall, from mucosa to serosa, and stimulated using electric field stimulation. The Ca^{2+} transients elicited spread through the untreated half of the bladder but not through the botulinum toxin type A (BoNTA)–treated side. Thus although BoNTA can be localized to one half of the bladder wall circumferentially by injections at two to four serosal sites (see Methods), it readily crosses from serosa to mucosa, at least in a 0.5-mm-thick mouse bladder.
M = mucosa, D = detrusor and S = serosa.

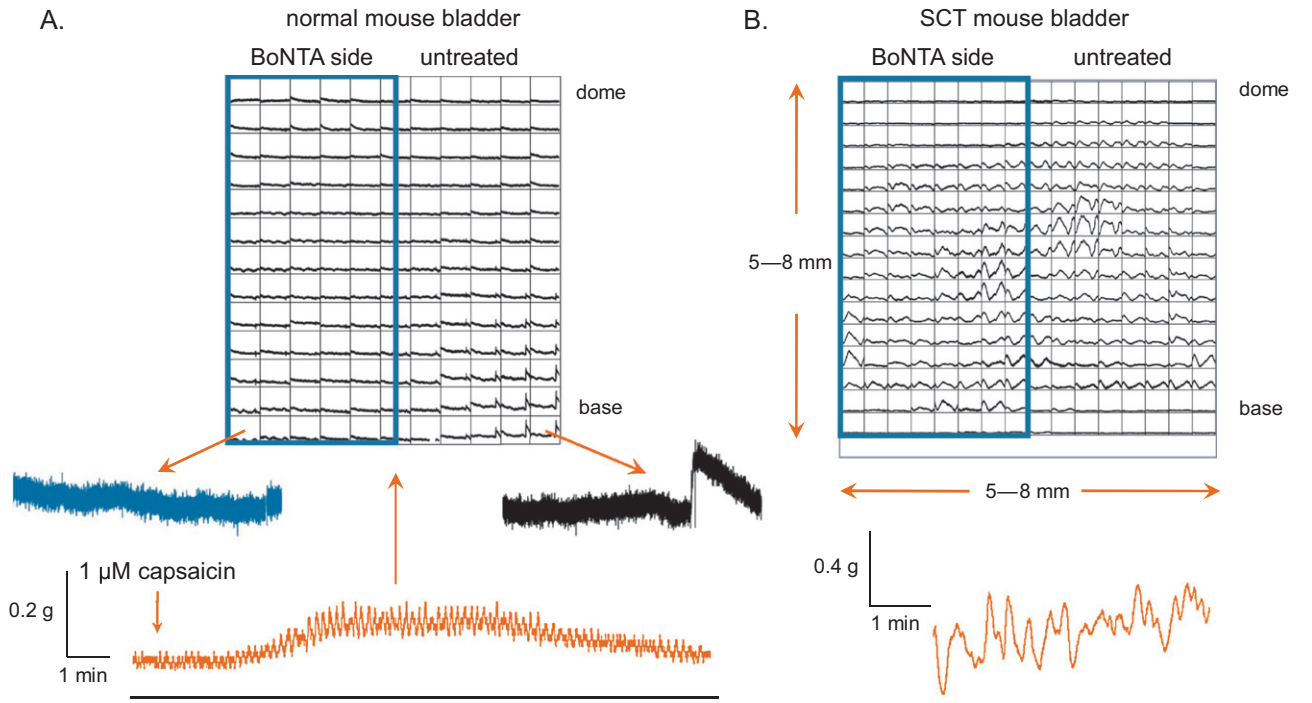

Fig. 5 – Optical mapping of capsaicin-evoked Ca^{2+} transients from the mucosal surface of control and spinal cord transected (SCT) mouse bladders treated with botulinum toxin type A (BoNTA). (A) Capsaicin (1 μM) caused an increase in baseline and contractile amplitudes in control mouse bladders. Optical mapping demonstrated that capsaicin-evoked Ca^{2+} transients only occurred in the untreated half of the bladder smooth muscle. A second dose of capsaicin did not evoke transients (not shown), presumably due to the depletion of neuropeptides and/or desensitization of transient receptor potential vanilloid-1 channels, demonstrating that the muscle signals are due to neuropeptides released from afferent terminals. (B) Intrinsic contractile activity was not inhibited by BoNTA treatment in SCT or control (not shown) mouse bladders.

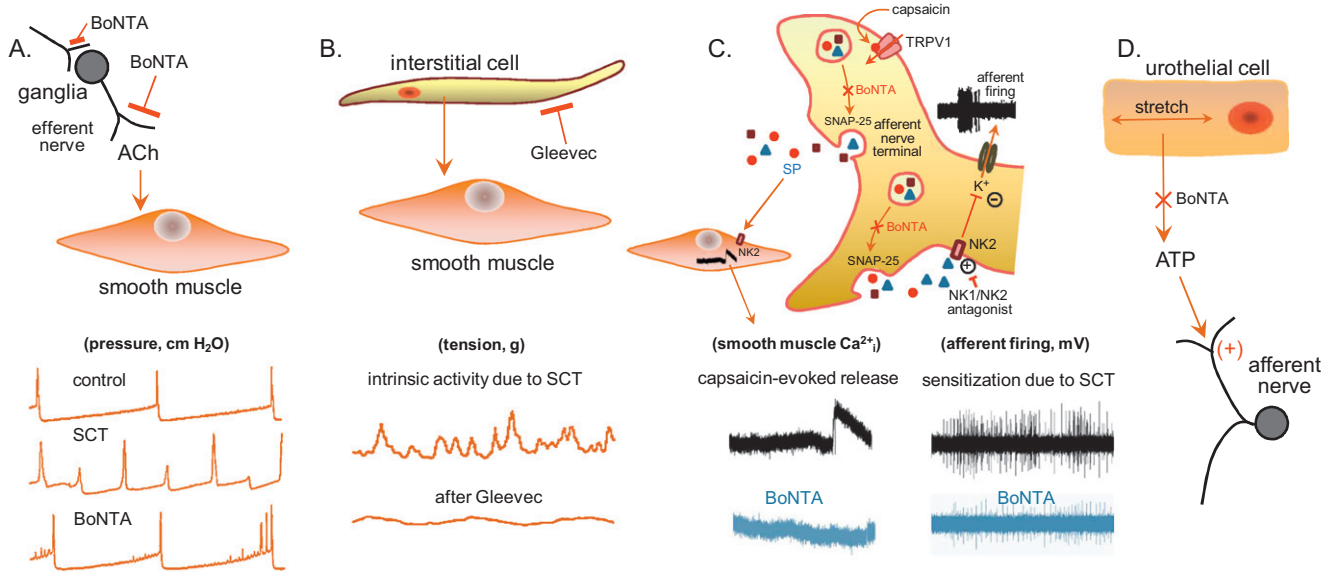

Fig. 6 – Schematic of different cell targets and mechanisms of action of botulinum toxin type A (BoNTA) in the urinary bladder. (A) Efferent nerve terminals: BoNTA can affect transmitter release from pre- and postganglionic parasympathetic nerve terminals. In the bladder, inhibition of acetylcholine (Ach) release from efferent nerves reduces detrusor contractility during micturition. This can be reflected in cystometric measurements as a decrease in reflex bladder contractions and intradetrusor pressures, and an increase in intercontractile intervals. (B) Interstitial cells: These cells are believed to promote intrinsic contractile activity of the detrusor, especially under pathologic conditions such as spinal cord injury or bladder outlet obstruction. (C) Afferent nerve terminals: BoNTA can reduce afferent sensitization by inhibiting neuropeptide release and decreasing firing frequency. (D) Urothelium: Urothelial cells release various factors including adenosine triphosphate (ATP) in response to stretch. Increased urothelial ATP release in pathology may contribute to afferent sensitization by acting on $P2X_2/P2X_{2/3}$ receptors. Because ATP release occurs through a vesicular mechanism, BoNTA may inhibit its release. NK = neurokinin.

4. Discussion

This study demonstrates that 48 h after the injection of BoNTA into the walls of control or SCT mouse bladders, afferent activity evoked by mechanical or chemical stimulation, or efferent activity evoked by electrical stimulation of the spinal nerves, was suppressed. However, intrinsic contractile activity was unaffected, suggesting this activity is nonneuronal and therefore not a target for BoNTA; this activity was inhibited by Gleevec (Fig. 6B). In addition, the effect of BoNTA did not appear to be limited to the sites of injection because tissue responses were markedly reduced throughout the layers of the bladder wall (Fig. 4).

The effect of BoNTA in decreasing afferent nerve firing induced by mechanical stimuli (stretch or intrinsic contractions) may involve multiple mechanisms. It has been demonstrated that NK receptor agonists inhibit K^+ currents in capsaicin-sensitive dorsal root ganglion neurons [22]. Assuming that these receptors and currents are also present in the nerve terminals, auto feedback stimulation would result in depolarization and enhanced afferent firing. BoNTA could decrease this firing by inhibiting the autoactivation of afferent NK2 receptors in rodents and NK1 receptors in humans (Fig. 6C). Alternatively, one could argue that BoNTA limits trafficking of voltage-gated Ca^{2+} channels to the membrane in afferent terminals [23], thereby limiting depolarization and afferent firing.

BoNTA attenuated contractile and Ca^{2+} activity in response to electric stimulation (field and direct nerve; Figs. 3 and 4). It is unlikely that there could have been a direct inhibition of the detrusor smooth muscle because BoNTA requires the SV2 protein to be transported inside a cell. In addition, we used stimulation parameters that we determined to preferentially depolarize nerves rather than smooth muscle cells (see Methods). Therefore, the inhibitory effects of BoNTA observed are neutrally mediated. The dosage used in this study is approximately five times that given clinically [8], so it is expected there would be a greater degree of parasympathetic inhibition. To determine if BoNTA had a direct effect on afferent-mediated neuropeptide release, we initially performed electrical stimulation in the presence of atropine and pyridoxal-phosphate-6-azophenyl-2′-4′-disulfonate (PPADS) to block muscarinic and purinergic receptors (not shown). As further confirmation that these electrically evoked Ca^{2+} transients resulted from neuropeptides released from afferents, we stimulated bladder tissues with capsaicin.

The suppression of capsaicin-induced Ca^{2+} signals in bladder smooth muscle provides further evidence that BoNTA blocks the release of neuropeptides from TRPV1-expressing afferent nerves. Capsaicin acts intracellularly on these channels to increase cytosolic Ca^{2+} and release neuropeptides (eg, substance P) that can activate neurokinin receptors in smooth muscle to elicit contractile activity. The effect of BoNTA to block the capsaicin responses is attributable to a direct action on afferent nerves because bladder smooth muscle does not contain SV2 receptors and is therefore not a site of action for BoNTs. Under pathologic conditions, bladder sensory nerves fire more frequently, thus increasing the likelihood of BoNTA binding to afferent SV2 proteins. A number of studies have examined the potential of targeting BoNTA to afferent nerves through modification of its protein structure [24,25], which would offer increased efficacy and a reduction in adverse side effects (eg, urinary retention).

Finally, the urothelium is a source of stretch-induced ATP release [26,27] that may act on $P2X_2/P2X_{2/3}$ receptors [28,29] on afferent nerves to promote their sensitization and increase bladder hyperreflexia [30]. Because urothelial ATP release is vesicular and SNAP-25 (pers. comm., L. Birder, Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA) and SV2 [31] have been identified in human urothelial cells, BoNTA may also act therapeutically on the urothelium (Fig. 6D).

5. Conclusions

BoNTA appears to elicit its suppressant effects on neurogenic detrusor overactivity in mice via the inhibition of neurotransmitter release from afferent as well as efferent nerve terminals without altering the intrinsic contractile activity of the detrusor. This could partly explain the effects of BoNTA treatment in reducing sensory symptoms in patients with bladder dysfunction.

Author contributions: Anthony J. Kanai had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Kanai.
Acquisition of data: Ikeda, Zabbarova, McCarthy, Kanai.
Analysis and interpretation of data: Birder, de Groat, Kanai.
Drafting of the manuscript: Ikeda, Zabbarova, Kanai.
Critical revision of the manuscript for important intellectual content: Birder, de Groat, Hanna-Mitchell.
Statistical analysis: Ikeda, Zabbarova, McCarthy, Kanai.
Obtaining funding: Kanai.
Administrative, technical, or material support: None.
Supervision: Kanai.
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Effect of Botulinum Toxin A on Urothelial-Release of ATP and Expression of SNARE Targets Within the Urothelium

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Aims: Botulinum neurotoxin serotype A (BoNT/A) has emerged as an effective treatment of urinary bladder overactivity. Intravesical lipotoxin (BoNT/A delivery using liposomes), which may target the urothelium, is effective in blocking acetic acid induced hyperactivity in animals. The objective of this study was to assess the possible site of toxin action within the urothelium. **Methods:** We examined expression of the toxin receptor (SV2) and its cleavage targets (SNAP-25 and SNAP-23) within urothelium as well as effects of the toxin on mechanically evoked release of ATP from cultured rat urothelial cells. ATP release was measured using the luciferin-luciferase assay; we examined expression of SNAP-23 and -25 in urothelial cells and mucosa of rat and human bladders. **Results:** BoNT/A (1.5 U; 1–3 hr) blocked hypotonic evoked release of urothelial ATP, without affecting morphology. The expression of protein targets for BoNT/A binding (SV2) was detected in human and rat bladder mucosa and catalytic action (SNAP-23, -25) in urothelial cells and mucosa (differed in intensity) from rat and human bladder. Incubation of cultured (rat) urothelial cells with BoNT/A decreased expression levels of both SNAP-23 (44%) and SNAP-25 (80%). **Conclusions:** Our findings reveal that the bladder urothelium expresses the intracellular targets and the binding protein for cellular uptake of BoNT/A; and that the toxin is able to suppress the levels of these targets as well as hypotonic-evoked ATP release. These data raise the possibility that intravesical treatment with BoNT/A suppresses bladder reflex and sensory mechanisms by affecting a number of urothelial functions including release of transmitters. *Neurourol. Urodynam. 34:79–84, 2015.* © 2013 Wiley Periodicals, Inc.

Key words: SNAP-25; SNAP-23; bladder mucosa; exocytosis

INTRODUCTION

The intramural injection of BoNT/A into the urinary bladder has proven to be a useful therapeutic approach for the treatment of various bladder disorders including refractory idiopathic detrusor overactivity as well as neurogenic detrusor overactivity.[1,2] Following uptake into nerve terminals BoNT/A is thought to affect bladder function by targeting intracellular proteins involved in exocytosis and then suppressing the release of transmitters from intramural nerves.

Though most of the studies on mechanism of action have focused on bladder nerves and change in neural control of the smooth muscle, other cellular targets may also be involved in the therapeutic effects of the toxin. Recent evidence has suggested that BoNT/A may act in part by targeting afferent mechanisms.[3,4] This may be due to a direct effect on afferent nerves or an indirect effect to block release of transmitters from the epithelial cells that line the bladder wall (i.e., the urothelium). Transmitters released from the urothelium are likely to alter the activity of underlying bladder afferent nerves or spontaneous activity of smooth muscle to alter bladder sensations.[5]

In motor neurons, BoNT/A cleaves the SNARE protein, SNAP-25, which plays a role in release of acetylcholine, thereby suppressing neurotransmission and preventing muscle contraction.[6] While there is also evidence for an effect on urothelial-release of mediators,[1,7] there are conflicting histological/anatomical results regarding the expression of protein targets for this toxin in the urothelium.[8] The purpose of this study was to assess the effect of BoNT/A on mechanically evoked ATP release from isolated urothelial cells and to examine the expression of its binding site (SV2) and SNARE proteins, SNAP-23 and SNAP-25, in human as well as rodent mucosa.

METHODS

Tissue and Urothelial Cell Preparation

All procedures were conducted in accordance and approval by both Institutional Animal Care and Use Committee and Institutional Review Board Committee Policies at the University of Pittsburgh. For immunoblot preparation, urinary bladder urothelium/lamina propria or "mucosa" (mechanically stripped from the underlying connective and smooth muscle tissues) from Harlan Sprague Dawley rats, aged 2–4 months, or de-identified superficial human biopsies from asymptomatic subjects were homogenized in HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose and 10 mM HEPES, pH 7.4 containing complete protease inhibitor cocktail, 1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO,

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1:100). After centrifugation (13,000*g*, 15 min), the membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris-HCl (pH 7.6) and 0.5% Triton X-100 and the same concentration of protease inhibitor as above. The suspensions were incubated on ice and centrifuged (13,000*g*; 15 min at 4°C) and protein concentrations were determined (Pierce BCA protein assay, Thermo Scientific, Rockford, IL).

Rat or human urothelial cell cultures were prepared as previously described.[9,10] Urinary bladders were removed and placed in cold MEM (Invitrogen, Carlsbad, CA) with HEPES (2.5 g/L, Sigma) and penicillin/streptomycin/fungizone (PSF; 1%; Invitrogen). The bladder was cut open to expose the urothelium, and incubated in dispase (2.0 mg/ml, Invitrogen) overnight at 4°C. Urothelial cells were gently scraped from the underlying tissue, placed in trypsin (0.25% w/v; 5–15 min, Invitrogen) triturated, suspended in MEM containing 10% FBS (Invitrogen) and centrifuged (416*g*; 15 min). For human biopsies, cultured cells were prepared according to previously published methods.[10] In brief, following dissociation in trypsin and resuspension in MEM (same as above), the supernatant was removed, and cells (rat or human) were suspended in PCT bladder epithelium medium (CNT-16, CELLnTEC, Bern, Switzerland). The cells were centrifuged again, resuspended in the same medium, plated on collagen-coated glass coverslips at 20×10^4 cells/ml and utilized within 18–72 hr following plating.

Antibodies

The SNAP-25 antibodies were from Covance (Emeryville, CA; SMI-81), Research and Diagnostic Antibodies (Las Vegas, NV; MC-6050), and Synaptic Systems (Gottingen, Germany). The rabbit polyclonal antibody to SNAP-23 and immunizing peptides for SNAP-23 and -25 were from Synaptic Systems. The mouse monoclonal antibody to SV2 (developed by Dr. Kathleen M. Buckley) was obtained from the Developmental Studies using a Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Iowa City, IA. Secondary goat anti-rabbit HRP and goat anti-mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and Becton Dickinson (Franklin Lakes, NJ), respectively. Loading control, rabbit anti-β-actin was from Abcam (Cambridge, MA). Z0-1 antibody was from Invitrogen, DAPI from EMD Millipore (Billerica, MA), DAPI from Molecular Probes (Eugene, OR) and cytokeratin antibodies from Dako Cytomation (Carpinteria, CA); fluorochrome-labeled secondary antibodies were obtained from Abcam.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from intact rat bladder mucosa by homogenization in Trizol (Invitrogen). RNA purification was performed using Qiagen RNeasy mini kit including an on column DNase Digestion step. RNA samples were reverse transcribed (RT) with an oligo (dT) primer by Superscript II (Invitrogen). RT-controls were performed for each sample by omission of the enzyme. Polymerase chain reaction (PCR) amplification was conducted using 1.25 units of platinum Taq DNA Polymerase (Life Technologies) using primer pairs for the following genes: synaptosomal-associated protein 23 (SNAP-23), synaptosomal-associated protein 25 (SNAP-25) and isoforms of the synaptic vesicle glycoprotein 2 (SV2-A, SV2-B, and SV2-C).Primer sequence; product size; and gene accession numbers were:

SNAP-23: forward: caccaacaagaatcgcattg; reverse: cccttctcgcacagtctttc; 150 bp; NM_022689

SNAP-25: forward: caaagatgctgggaagtggt; reverse: gggggtgactgactctgtgt; 181 bp; NM_030991

SV2-A: forward: tttgggggagtcagtattgc; reverse: cccacaaaggacgtgaagat; 155 bp; NM_057210

SV2-B: forward: agctgtatcccaccaaccag; reverse: agagaagcagcagccagaag; 145 bp; NM_057207

SV2-C: forward: gtacaaggaccgcagagagc; reverse: taacacaaagcccaccacaa; 181 bp; NM_031593

Amplification products were visualized on a 1.3% agarose gel with ethidium bromide. Empty RT^- lanes confirmed purity of the RNA isolation (absence of genomic DNA).

Immunoblot (SNAP-23, SNAP-25, and SV2)

Lysates from each sample were denatured in SDS–PAGE sample buffer (all except SV2 were treated at 100°C for 5 min) and separated on an SDS–PAGE gel using a standard Western protocol. Proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% Milk TBS-T (1 hr), rinsed in TBS-T, and incubated (overnight at 4°C) with primary antibody diluted in 5% Milk TBS-T (SV2 and SNAP-23) or 2% normal goat serum TBS-T (SNAP-25). The membranes were then incubated with secondary antibody for 1 hr in 5% Milk TBS-T, developed with ECL Plus (Amersham, Piscataway, NJ) and exposed to film. The volume of each band was determined using a Personal Densitometer SI (Molecular Probes, Carlsbad, CA). The membranes were stripped (membrane recycling kit from Alpha Diagnostic International, San Antonio, TX) and reprobed overnight with rabbit anti-β-actin as a loading control. Bands for SNAP-23 (23 kDa) and SNAP-25 (75 kDa) were blocked by incubation with the immunizing peptide (1 mg/ml). A single immunoreactive band was observed for SV2 (95 kDa) and β-actin (43 kDa); a competing peptide was not commercially available for these antibodies.

Immunohistochemistry/Immunocytochemistry (SNAP-23; SNAP-25; Z0-1)

Excised bladders were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek OCT, Sakura Finetek, CA), snap frozen and stored at −80°C. Serial cryosections (6 μm) were mounted onto microscope slides, post-fixed with 4% paraformaldehyde and washed in phosphate buffered saline (PBS). Primary cultured urothelial cells, grown on glass coverslips, were fixed with 2% paraformaldehyde and washed in PBS. Tissue sections or cells were incubated with permeabilizing/blocking solution (0.5% Triton X-100 and 10% goat serum in PBS) before incubation (24–48 hr at 4°C) with primary antibodies in blocking solution (10% goat serum in PBS). Cells were incubated with monoclonal anti-cytokeratin 17 (basal urothelial cell marker; Dako) and polyclonal anti-zona occludens-1 (ZO-1; a tight junctional marker; EMD Millipore) at a 1:200 dilution. Tissue sections were incubated with rabbit polyclonal primary (SNAP-23 or SNAP-25, 1:100, Synaptic Systems) antibodies; control experiments were done using antibodies blocked by incubation with the immunizing peptide (1 mg/ml). Co-localization studies were also conducted using monoclonal anti-cytokeratin 17 (1:500). Tissue and cells were washed with PBS before incubation with the appropriate secondary antibodies. Cells were incubated (2 hr at room temperature) with fluorophore-tagged secondary antibodies: Alexa Fluor 488 Donkey Anti-Mouse and (green) and Alexa Fluor 555 Donkey Anti-Mouse (red) (1:700; Invitrogen). Tissue sections were incubated with donkey anti-rabbit biotin (1:1,000, Abcam) for 2 hr, washed, and incubated with Neutravidin FITC (1:1,000, Abcam).

Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole, dilactate; Molecular Probes, Eugene, OR). Tissue or cells were treated with ProLong Gold antifade reagent (Invitrogen) and imaged on a BX-62 Olympus upright fluorescent microscope using CImaging software (Hamamatsu Photonics, Sewickley, PA). Background immunofluorescence was assessed in the absence of primary antibodies and secondary only.

ATP Release Assay

ATP release was conducted as per previously published methods.[9] In brief, a perfusate of 100 µl of HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM Glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) was collected every 30 sec. Background release of ATP from the cultures was measured for 15–20 min prior to the test stimulus (HBSS with 50% reduction of NaCl). ATP measurements were calculated based on the luciferin-luciferase reaction using a standard curve (Adenosine Triphosphate Assay Kit, Sigma; Glomax 20/20 Luminometer, Promega). Data were analyzed by calculating the area under the curve during each portion of the experiment.

Statistical Analysis

Data were graphed in GraphPad Prism 5 and analyzed by a paired Students *t*-test. Data were considered significantly different when $P < 0.05$.

RESULTS

Expression of the BoNT/A receptor, SV2, as well as the SNARE proteins, SNAP-23 and SNAP-25, was detected in urinary bladder mucosa (ex vivo). Using RNA extracted from rat mucosal tissue and gel electrophoresis, positive bands were obtained for SNAP-23, SNAP-25, and all three SV2 isoforms (SV2-A, SV2-B, SV2-C) with the SV2C isoform exhibiting the greatest expression (Fig. 1A). Protein expression for SV2, SNAP-23, and SNAP-25 was also evident in rat mucosa and in human mucosal biopsies taken from asymptomatic controls. Positive bands for SNAP-23 and SNAP-25 were also observed in proteins extracted from urothelial cells isolated from rat as well as human urinary bladder and SV2 in rat mucosa (Fig. 1B). We also have observed a difference in the immunoblot intensity between mucosal samples and cultured urothelial cells, though the reasoning for these differences are not known. The protein band for both SNAP-23 and SNAP-25 for mucosa and cells was completely blocked by incubation of the antibodies with the immunizing peptide, demonstrating specificity of the antibodies.

Immunocytochemical studies revealed expression of both SNARE proteins within the rat urothelium. Co-localization experiments for urothelial cytokeratin 17 (basal cells) revealed that SNAP-25 was observed throughout the urothelium (Fig. 1C) while SNAP-23 was expressed mainly within the superficial or apical layer (Fig. 1D). Omission of primary antibodies from the incubation buffer as well as incubation of the antibodies with

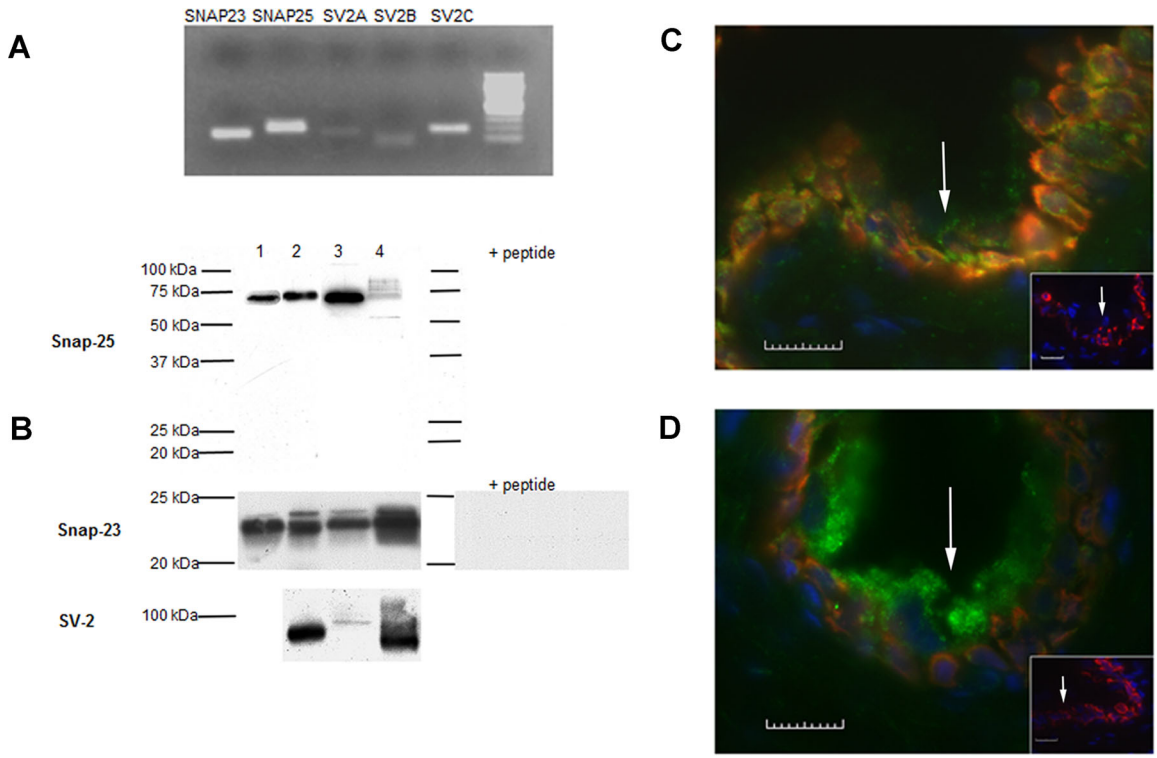

Fig. 1. The synaptosomal-associated protein-23 (SNAP-23), synaptosomal-associated protein-25 (SNAP-25) and synaptic vesicle protein 2 (SV2) are expressed in urinary bladder urothelium. **A**: Electrophoresis of PCR product on a 1.3% agarose gel with ethidium bromide staining showing positive mucosal expression in the rat of SNAP-23, SNAP-25 and synaptic vesicle (SV) glycoprotein isoforms: 2A, 2B, and 2C (SV2-A, SV2-B, SV2-C). **B**: Using immunoblot analysis is the protein expression for SNAP-23 (23 kDa), SNAP-25 (75 kDa), and SV2 (95 kDa). Each lane represents samples from the same source: human urothelial cells (lane 1), human mucosa (lane 2), rat urothelial cells (lane 3) and rat mucosa (lane 4). The protein expression for both SNAP-23 and SNAP-25 was completely blocked by the immunizing peptide. **C**,**D**: Rat bladder cross-sections showing SNAP-25-immunoreactivity (IR, panel C, expressed throughout urothelium) and for SNAP-23-IR (panel D, expressed mainly in superficial or apical cells). Arrows in each panel indicate the epithelial surface. In both C and D inset panels show lack of SNAP-IR in controls following incubation with the corresponding immunizing peptide (calibration bar = 10 µm).

the immunizing peptide completely attenuated secondary antibody labeling (data not shown).

To explore the relative expression levels for both SNAP-23 and SNAP-25 in the mucosa (in human and rat), we stripped each blot and compared the relative expression patterns (normalized to β-actin; Fig. 2A). The experiments revealed a distinct difference in distribution in tissue from rat and human. SNAP-25 was by far the predominant protein in human tissue (93% of total) but the minor component (40%) in rat tissue. SNAP-23 was approximately 10-fold higher in rat versus human tissue and SNAP-25 was approximately 2.5-fold higher in human tissue.

We next used cultured urothelial cells from rat urinary bladder to examine the effect of botulinum toxin A (BoNT/A) on expression levels of SNAP-23 and SNAP-25 proteins as well as on release of ATP evoked by exposure to hypotonic solution. Incubation with BoNT/A (1.5 U/ml; for 1–3 hr) decreased the expression of both SNAP-23 (44% decrease as compared with control) and SNAP-25 (80% decrease as compared with control) (Fig. 2B).

Cultured urothelial cells released a low level of ATP in the absence of stimulation and exposure to hypotonic solution elicited a large increase in ATP (10.4 ± 2.6 nM; Fig. 2C). Incubation with BoNT/A (1.5 U; for 1–3 hr) completely blocked the hypotonic-evoked ATP release (Fig. 2C). Depletion of ATP stores by BoNT/A is unlikely to be the cause of the suppression of ATP release because mechanical disruption (lysis) of the cells at the end of the experiment resulted in ATP release which was not significantly different in treated and untreated cells (data not shown). Incubation of urothelial cells with the toxin for 24 hr did not result in any visible changes in cell morphology such as cell blebbing or loss of tight junction proteins (Fig. 2D).

DISCUSSION

By blocking release of multiple transmitters and/or reducing receptor expression (i.e., muscarinic, P2 × 3, TRPV1, EP4),[11–12] botulinum toxin A (BoNT/A) has the potential for relieving symptoms of overactive bladder as well as the hyperalgesia associated with other lower urinary tract disorders.[12–15] Though bladder afferent and efferent nerves have been proposed[4,8,16] as sites of action for BoNT/A and SV2 and SNAP-25 are expressed in these nerves, the mechanism of toxin action is likely to be more complex involving other cells within the bladder wall. Our findings reveal that urinary bladder urothelium expresses the synaptosomal proteins SNAP-25 and SNAP-23. The former is involved in release of transmitters from nerve cells while the latter is thought to be involved in non-neuronal secretory processes and is expressed in tissues rich in epithelial cells. The protein receptor for BoNT/A has been shown to be SV2 (in this study expressed in mucosa and rat UT cells) and some studies have indicated that specific isoforms (such as SV2C in phrenic nerves[17]) may be involved in mediating toxin uptake. Though all three SV2 isoforms are expressed in mucosa (with SV2-C at the highest levels), it is not yet known which SV2 isoforms are involved in the uptake of

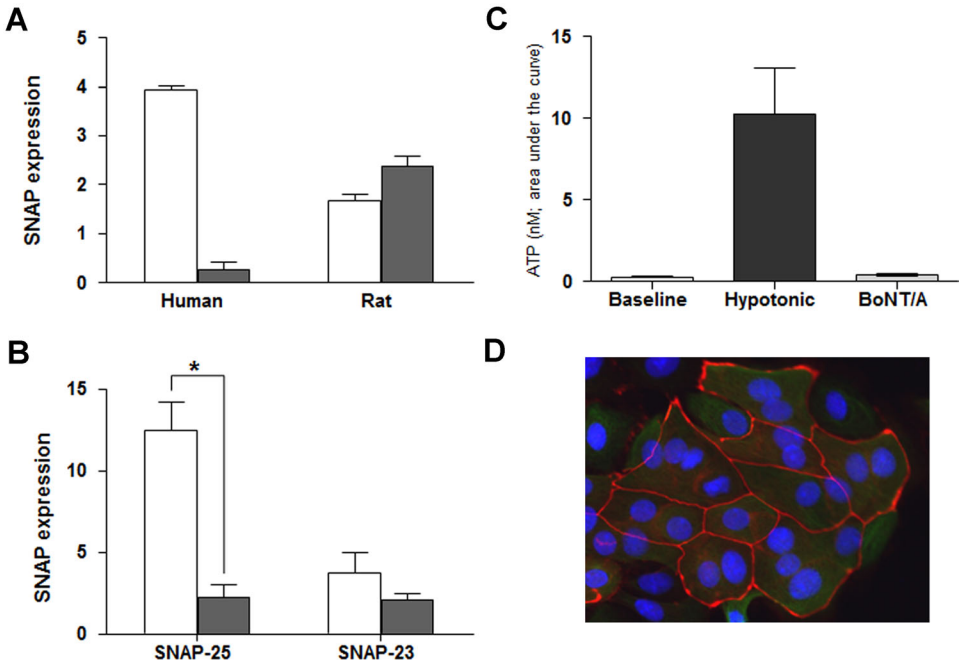

Fig. 2. The effect of BoNT/A on SNAP-25, SNAP-23 expression and ATP release. **A**: Summary of Western blot analysis showing protein levels of SNAP 25 (open bars) versus SNAP-23 (solid bars) in both human and rat mucosa. SNAP-25 levels in rat mucosa are less than half of the levels in human mucosa; while SNAP-23 levels in rat mucosa are 10-fold higher as compared to human. Three independent experiments were analyzed and are normalized to beta-actin. **B**: Levels of SNAP-25 and SNAP-23 protein in control rat urothelial cells (open bars) and urothelial cells treated with BoNT/A (1.5U, for 1–3 hr, solid bars). BoNT/A significantly decreased the expression of SNAP-25 ($P < 0.05$ as compared by unpaired Student's *t*-test). **C**: BoNT/A inhibits hypotonic-evoked release of ATP. Summary graph showing baseline release of ATP, the effect of hypotonic stimuli alone, and hypotonic-evoked release following treatment of cells with BoNT/A (1.5 U; 1-3 hr). Data are expressed as nM of ATP and were obtained from cultures prepared from at least 6 rats. Experiments were performed on at least $n = 6$ independent cultures from each animal. $P < 0.05$ as compared by unpaired Student's *t*-test. **D**: Prolonged exposure to BoNT/A does not induce deleterious changes in urothelial cell morphology. Shown is a representative fluorescent image of cultured rat urothelial cells 24 hr following incubation with BoNT/A. The cells were fixed (4% paraformaldehyde) and stained with the junctional marker ZO-1 (shown as red; primary rabbit and secondary antibody Cy3 goat-anti-rabbit IgG) and counter-stained with the nuclear marker, DAPI (shown as blue).

BoNT/A within urothelium. Though a previous study has reported the expression of SNAP-23 within the apical urothelial layer of rat urinary bladder,[18] to the best of our knowledge our results are the first to also show evidence of both SNAP-25 and SNAP-23 in both rat and human bladder urothelium as well as a relative difference in expression levels. Though the observed band size for SNAP 25 (75 kDa) in our preparations differs from the predicted value, one explanation for this difference includes the formation of SNAP25 containing protein complexes. Studies have shown the presence of multiple SNAP-protein complexes (identified from 40 to 120 kDa) that can form teterodimers and heterotrimers in various conditions in a number of (tissue and cellular) preparations including hippocampal slices.[19,20] Further, these types of complexes are likely to migrate at a different position than the predicted molecular weight of SNAP-25 (25 kDa) would suggest, and this could also reflect a number of protein changes (i.e., glycosylation or palmitolyation). Our findings were confirmed using urothelial cells as well as mucosa from rat and human biopsies.

SV2 as well as SNAP-25 is expressed in parasympathetic and sympathetic (VAChT and TH positive) efferent as well as afferent (CGRP positive) bladder nerves.[8] By inhibiting SNARE-dependent exocytotic processes, BoNT/A can prevent the release of transmitters (i.e., acetylcholine, norepinephrine, ATP) from both types of efferent nerves or release of neuropeptides (i.e., substance P, CGRP) from afferent nerves as well as suppress the translocation of various receptors and channels (TRPV1, $P2X_3$) to the plasma membrane.[1,3,18,21,22] In addition, recent studies have shown that BoNT/A injected in the bladder wall of the mouse urinary bladder can suppress afferent activity evoked by mechanical or chemical stimulation.[4] There is also evidence that BoNT/A treatment (in vitro) can normalize neuronal SNAP-25 expression that is altered in different pathologies.[23] There is ample precedent for expression levels of a large number of proteins (including SNAP) rapidly changing in a number of cell types. For example, it has been shown in motoneurons[24] that SNAP-25 levels decrease 1 hr after exposure to BoNT/A, which correlates and supports our findings that both SNAP-23 and SNAP-25 expression levels are significantly decreased in urothelial cells hours after exposure to the toxin. In parallel experiments, we also demonstrate that BoNT/A inhibits the release of ATP from cultured urothelial cells; a finding previously reported using intact urinary bladder.[7] Urothelial-release of ATP can stimulate purinergic receptors on nearby bladder afferents that can convey information about bladder filling or irritation to central nervous system.[25] The sensitivity of trafficking processes involving translocation of receptors,[26] membrane,[27,28] and other putative urothelial transmitters[29] during bladder filling remains to be elucidated.

The mechanism of action of BoNT/A is thought to involve binding to the membrane followed by internalization into the cytosol and finally inhibition of cellular processes involving exocytosis. In neurons, the toxin is thought to bind with vesicular proteins such as SV2 when vesicular membrane is exposed to the extracellular compartment during exocytosis.[1,30] In the urothelium a similar mechanism may mediate endocytosis of BoNT/A. We have evidence as also reported by others[31,32] that this type of binding does not elicit any adverse effects on cellular structure or function. However while the internalized toxin may remain in the terminals of neurons (though retrograde axonal transport to the CNS may also occur),[33,34] in polarized epithelial cells internalized toxin may distribute throughout the entire cell and be present within vesicles that mediate transcellular transport. In the epithelium of the small intestine the neurotoxin does not passively diffuse but can undergo transcytosis across cells to eventually reach the circulation and elicit systemic effects such as block of transmission at the neuromuscular junction.[35] In the urinary bladder, this type of transport may provide a way for the toxin to "escape" the lumen to reach cells underlying the urothelium thereby broadening its effects within the bladder wall and extending its duration of action. In this regard, the duration, formulation as well as the concentration of BoNT/A instillation may lead to changes in penetration (or trafficking) that could influence other cell types in the bladder wall.

Although previous studies have failed to detect the expression of SNAP-25 within the bladder mucosa,[8] the present experiments revealed the expression of SNAP-25 and SNAP-23 in rat and human urothelium as well as SV2 in rat and human mucosa. A difference in the immunoblot intensity between mucosal samples and cultured urothelial cells was observed, though the reasoning for these differences are not known. Though mucosal samples could contain other sources of SV2 and SNAP-25 protein (i.e., afferent and efferent nerve terminals), these sources should not exhibit positive signals using RT-PCR. While SNAP-23 is expressed in both neuronal and non-neuronal cells, it is possible that the difficulty in detection of non-neuronal SNAP-25 may be due to low expression levels, as reported for pancreatic endocrine cells.[36] While we did not examine SNAP-25 cleavage products in the present study, a number of variables may influence cleaved SNAP-25 content. Studies have shown that in some cases limited amount of SNAP-25 may actually be cleaved (in nerves innervating the detrusor) and identification of cleavage product may also be precluded by sensitivity of the detection.[37] Further, the use of SNAP-25 (for detection of neurotoxin activity) has been based on the endopeptidase activity of the L-chain of BoNT/A that enables the toxin to degrade the SNAP-25 protein in nerves. There is no information as to the activities of the toxin on exocytotic processes (including transmitter release) from urothelial cells. Consistent with previous studies[17] we also found that SNAP-23 is expressed mainly within the superficial or apical urothelium that may in part explain the higher expression of SNAP-23 in rat mucosa compared to isolated urothelial cells (containing mainly basal cells in the preparation). Though in some cell types the roles for specific fusion events have been linked to specific components of the SNARE machinery, it is unclear why urothelial cells express both SNAP-25 and SNAP-23 (which is also sensitive to BoNT/A)[38] and also how these SNAREs regulate urothelial fusion events. While SNAP-25 and SNAP-23 may be functionally equivalent, there is evidence that these two SNARE proteins may play a role in exocytotic processes having different calcium thresholds.[39] Thus it is possible that SNAP-25 may be linked with a higher rate of fusion events. In contrast, SNAP-23 may be involved in a constitutive release pathway in epithelial cells, involving docking and fusion of vesicles that occur at low or resting calcium concentrations.

CONCLUSIONS

Taken together, our findings suggest that the mechanism of action of BoNT/A in the urinary bladder may be more complex and likely to involve urothelial sites of action. Support for this also comes from a Phase I/II study in which clinical improvement was seen following intravesical instillation of BoNT/A with DMSO[40] and a promising effect on irritant-induced bladder hyperactivity noted in animal models using intravesical instillation of BoNT/A encapsulated in liposomes.[41] It is likely that BoNT/A inhibition of transmitter release and other exocytotic processes in urothelial cells may be complex. This may involve additional mechanisms such as inactivation of

proteins (i.e., synaptotagmins)[42] that regulate exocytotic events.

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Do We Understand Any More About Bladder Interstitial Cells?—ICI-RS 2013

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Aims: To present a brief review on discussions from "Do we understand any more about lower urinary tract interstitial cells?" session at the 2013 International Consultation on Incontinence-Research Society (ICI-RS) meeting in Bristol, UK. **Methods:** Discussion focused on bladder interstitial cell (IC) subtypes, their localization and characterization, and communication between themselves, the urothelium, and detrusor smooth muscle. The role of ICs in bladder pathologies and new methods for studying ICs were also addressed. **Results:** ICs have been studied extensively in the lower urinary tract and have been characterized based on comparisons with ICs of Cajal in the gastro-intestinal tract. In fetal bladders it is believed that ICs drive intrinsic contractions to expel urine through the urachus. These contractions diminish postpartum as bladder innervation develops. Voiding in human neonates occurs when filling triggers a spinal cord reflex that contracts the detrusor; in rodents, maternal stimulation of the perineum triggers voiding. Following spinal cord injury, intrinsic contractions, and spinal micturition reflexes develop, similar to those seen during neonatal development. These enhanced contractions may stimulate nociceptive and mechanosensitive afferents contributing to neurogenic detrusor overactivity and incontinence. The IC-mediated activity is believed to be initiated in the lamina propria by responding to urothelial factors. These IC may act syncytially through gap junction coupling and modulate detrusor activity through unknown mechanisms. **Conclusion:** There has been a great deal of information discovered regarding bladder ICs, however, many of their (patho)physiological functions and mechanisms are still unclear and necessitates further research. *Neurourol. Urodynam. 33:573–576, 2014.*

Key words: detrusor overactivity; interstitial cells; lamina propria; LUTS

INTRODUCTION

It has been demonstrated that newborn rats,[1] pigs,[2] and human infants[3] have bladder ICs with similar morphological and immunological properties to their adult counterparts. Bladder ICs have been defined according to ultrastructural features[4–6] and are distinct from cells with similar morphologies including myofibroblasts and telocytes. These ICs are important for the development and function of the urinary bladder as long term treatment with the *c*-kit inhibitor, imatinib, results in impaired bladder development, and contractility.[1] It has been suggested that intrinsic activity in neonatal and pathological bladders is associated with an increase in IC number and coupling.[7] This is supported by findings that patients with overactive bladders have both increased numbers of ICs and enhanced sensitivity to imatinib.[8] Accordingly, ICs have an important role in bladder development, function and pathology, and merit further research. The information presented is a brief summary of current knowledge on bladder ICs as discussed at the ICI-RS 2013 meeting. For more comprehensive reviews refer to.[8,9]

IC SUBTYPES, LOCALIZATION, AND CHARACTERIZATION

Bladder ICs were initially identified by their morphological similarity to ICs of Cajal (i.e., spindle shaped with extensive processes) and selective responses to nitric oxide donors.[10,11] The similarities has raised the possibility of bladder ICs also being pacemakers like ICs of Cajal to regulate the contractile activity of smooth muscle. Although, ICs of Cajal and bladder ICs appear to share morphological and receptor expression characteristics, they appear to differ functionally.

There have been progressively more cell surface markers identified on bladder IC, including *c*-kit tyrosine receptor kinase,[12] platelet-derived growth factor α[13] and β[14] receptors (PDGFRα and β), and CD34[15] all of which are markers for hematopoietic stem cells. This, along with differential localization of ICs in the bladder wall, has brought about the concept of distinct IC subtypes mediating various processes including tissue regeneration and remodeling (myofibroblasts and telocytes), signal transduction, or pacemaker activity (ICC-like cells, Table I). The use of novel markers is crucial to identifying new IC subtypes. PDGFRα is co-expressed with *c*-kit and vimentin-positive cells in rat bladders[9]; and nucleoside triphosphate diphosphohydrolyase2 (NTPDase2) and anoctamine-1 (Ca^{2+} activated chloride channel, Ano1) co-label CD34 positive cells in mouse bladders.[14] Pacemaker-like activity has been associated with c-kit[16] or PDGFRα[17] positive ICs. The relationships of other IC markers to specific functions have yet to be determined.

Conflict of interest: none.
Karl-Erik Andersson led the peer-review process as the Associate Editor responsible for the paper.
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TABLE I. General Classification of Interstitial Cell-Like Cells in the Urinary Bladder, Including Potential Functions and Cell–Cell Interactions

Cell types	Functions	Interactions
Myofibroblasts	Muscle progenitor	Smooth muscle, urothelium, nerve terminals
Interstitial cells of Cajal-like	Pacemaker cell	Smooth muscle, urothelium, nerve terminals
Telocytes	Inter-cellular communication	Urothelium, nerve terminals, capillaries, inflammatory cells

Gene (splice variants) and protein (isoforms) signatures are needed to characterize IC populations. ICs in lamina propria and detrusor have different ionic currents[18,19] suggesting these cells may have specialized functionality related to their location. However, there are also species and tissue-specific differences, for example, three Ano1 exons are expressed in human male bladders.[20] This is important as pharmacological agents might discriminate between receptor/channel isoforms and have implications for the development of animal models. Of interest, however, is that *c*-kit mutant mice and rats have unaffected bladder IC networks.[21] Ano1 knockout mice have a very severe gut phenotype, which lacks peristalsis[22]; but quantitative analysis of the gut IC network structure properties revealed no changes between wild type and knockout mice.[23] There are no reports on bladder ICs in this animal model. It is unknown, which protein isoform, network characteristics in pathophysiology, and compensatory mechanisms might be behind the lack of visible changes. IC markers have also been identified in human bladder biopsies[24,25] and alterations in ICs have been linked to pathological conditions.[26] However, there are little to no functional studies using human tissue samples and this is a deficit that needs to be addressed.

The location of ICs could also be significant in respect to their function. Bladder ICs are located throughout the lamina propria, between detrusor muscle bundles (intra-muscular), within muscle bundles (inter-muscular)[27] and around blood vessels (perivascular)[6] (Fig. 1). These ICs may be involved in regulating the activity of smooth muscle or nerve terminals in their respective locations.[18,28,29] Isolated lamina propria ICs differ from those of the muscle layer when challenged with various receptor agonists, for example, lamina propria ICs readily respond to ATP but not ACh.[18] This difference has led to the concept of lamina propria ICs being potential transducers for urothelial signaling factors (e.g., ATP) to the detrusor. Similarly muscle ICs may more readily respond to neurotransmitters that mediate detrusor contractility (e.g., ACh) due to their proximity to efferent terminals.[29]

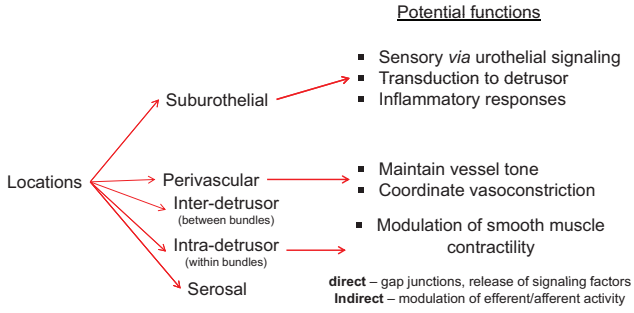

Fig. 1. Localization of interstitial cells in the urinary bladder and their hypothesized functional significance.

IC CELL–CELL INTERACTIONS

ICs in the bladder lamina propria (i.e., suburothelium) and the detrusor layer are characterized by connexin (Cx) labeling, in particular of Cx43,[14,30] and in lamina propria ICs are associated with gap junctions between adjacent cells. ICs also demonstrate large, spontaneous transient inward currents mediated in part through Ca^{2+}-activated Cl^- currents,[18] possibly Ano1,[14] and activated by exogenous agents such as ATP and other purinergic agonists, or reduction of extra-cellular pH.[31] Thus, it is likely that electrical signals can flow between cells and an IC network can act as a functional syncytium. Moreover, the ability to propagate electrical signals may be enhanced in conditions associated with detrusor overactivity as the number of ICs is greatly increased.[31,32] An intriguing observation is that physical abutment of adjacent ICs, without the formation of gap junctions, augments the electrical activity of individual cells[33]—this enhancement was attenuated by imatinib.[31] The exact mechanism for the enhancement is unclear but demonstrates the importance of cell–cell contact in signal amplification through the IC networks.

Greater connectivity in an interstitial network may thus appear as larger, better-coordinated spontaneous electrical, or contractile activity across the bladder wall. Electrical signals and Ca^{2+} waves propagate from the suburothelium to the detrusor and this activity is augmented in detrusor overactivity.[31] Moreover, localized micromotions of the bladder wall are more sustained and of higher frequency in human subjects experiencing increased sensations on bladder filling.[34] Thus, in overactive bladders, ICs in the bladder,[35] and urethra[36] may act as pacemakers similar to ICs of Cajal in the gastrointestinal tract.[37]

ICS IN BLADDER PATHOLOGY

The role of ICs in normal bladder pathology is still unclear. However, in pathologies such as spinal cord injury, neurogenic bladder dysfunction, and partial bladder outlet obstruction (PBOO) there is prominent spontaneous detrusor contractions. Changes to bladder ICs in these conditions include; increased IC numbers,[7] morphologies and distributions,[38] and gap junction connectivity throughout the bladder wall,[7] as mentioned in the previous section. The enhanced coupling between ICs is believed to facilitate the coordinated stimulation of the detrusor to enhance intrinsic contractile activity and can lead to detrusor overactivity. The coupling of ICs may occur as a compensatory mechanism in response to a disruption of bladder innervation, similar to the situation in neonatal stages. Studies with neonatal rodent bladders have demonstrated large amplitude intrinsic detrusor contractions and voiding facilitated by a bladder–spinal cord reflex pathway.[39] It is hypothesized that ICs respond to bladder filling by enhancing the intrinsic detrusor contractions to trigger the spinal voiding reflex (Fig. 2). In situations such as spinal cord injury where neural input is disrupted, remodeling, and coupling of ICs could be a mechanism to aid voiding.

ICs may also be involved with sensory processing, a concept derived from their close approximation to afferent nerves.[40] Additionally ICs respond to a number of signaling factors including ATP,[18] ACh,[28] prostaglandins,[41] and NO,[11] but their functional roles are still unclear. Certain pathologies have been linked to increased release of urothelial signaling factors such as ATP (e.g., interstitial cystitis/painful bladder syndrome, spinal cord injury),[42,43] which could potentiate IC activity. Indeed, it has been demonstrated that there is increased $P2X_3$[44] and possibly muscarinic M_3-receptors in bladder ICs following

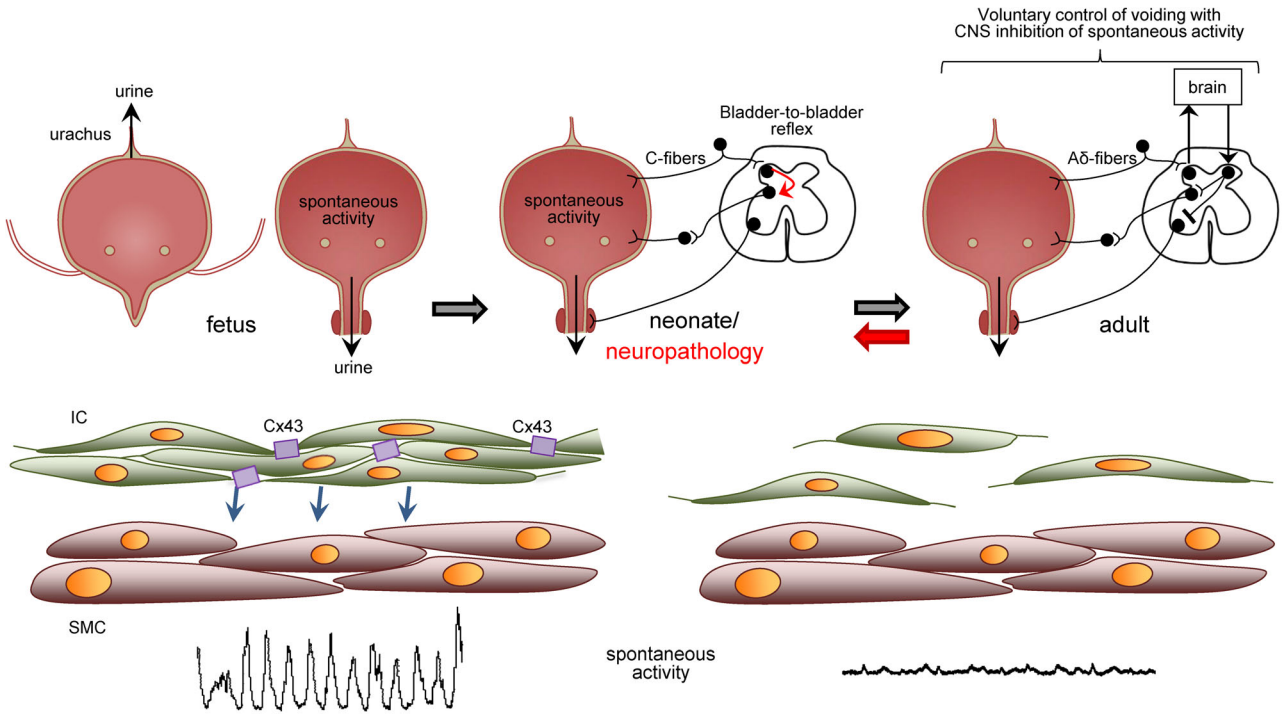

Fig. 2. Proposed mechanism for interaction of IC-mediated spontaneous contractions and spinal reflex pathways. Urine is expelled from the fetal bladder via the urachus in utero. In neonates, IC forms a syncytium through gap junctions that allow for coordinated activation of detrusor smooth muscle. These large magnitude contractions can stimulate mechanosensitive afferents that activate a bladder-to-bladder spinal pathways that triggers micturition. The spinal reflex pathway is lost in adulthood and voiding becomes regulated entirely by the CNS. Along with neural remodeling in the spinal cord, there is decreased connectivity between bladder IC and spontaneous activity. In neuropathologies, a spinal reflex loop similar to that in neonates can form, along with increased bladder IC connectivity. This may be a initially compensatory mechanism to allow voiding in the absence of CNS input. However, in conditions such as spinal cord injury this can lead to detrusor sphincter dyssynergia due to uncontrolled neural remodeling.

PBOO.[45] Thus it could be hypothesized that the combined effect of increased receptor expression and signaling factor release could further potentiate IC activity and exacerbate detrusor overactivity and/or alter sensory processing.

NEW METHODS FOR STUDYING IC

Novel approaches, including subtype characterization, development of animal models, pharmacological, and cell-interaction studies are used to shed light on the functional role of ICs and in turn their clinical relevance. Although these studies have yielded important information on the characteristics of these cells, there are still a number of unresolved questions regarding functionality, particularly their interactions with other tissues.

Studying cell-to-cell communications, while difficult in intact tissues, can be done using cultured cells. Optical mapping of confluent strips of ICs from normal rodent bladders placed between strips of urothelial and smooth muscle cells may be used to determine their role in urothelial to smooth muscle communication. When the urothelial cells were stretched by osmotic distension, signals propagated from the urothelial to smooth muscle. When the ICs were removed, the smooth muscle did not respond to stretch.[46] In addition, ICs from spinal cord transected rodents exhibited rhythmic Ca^{2+} transients, which could directly communicate with smooth muscle causing it to contract.[42] Such in-line culture based systems allow mapping of information flow between cell types in a controlled manner. In addition, different IC subtypes could be isolated using cell sorting methods targeting different surface markers. Cell sorting has been utilized to separate ICs from human mucosal samples for culturing purposes[47] and this technique could be further developed to isolate based on other IC surface markers.

Crucial for understanding the role of ICs in bladder sensation is the determination of whether they directly interact with afferent nerves in the bladder wall. This can be studied using pseudorabies viruses that express the Ca^{2+} sensor, GCaMP.[48] These viruses can be injected into the L6–S2 (pelvic) and T13–L2 (hypo-gastric) ganglia to label their afferent projections to the bladder wall. Studies using this approach have demonstrated that afferent nerves communicate with urothelial cells but not ICs (preliminary data presented by the Kanai lab at the ICI-RS 2013 meeting).

SUMMARY AND RESEARCH QUESTIONS

ICs potentially have a key role in driving the symptomology of various bladder disorders, but the exact nature of IC function under normal physiology and pathophysiology has yet to be elucidated. Further experimental evidence is required for associating specific IC markers/subtypes or ICs from different parts of the bladder wall with specialized functionality, and this would have to be followed by investigations in bladder pathology models. Additionally, the characteristics of isolated cells may not necessarily reflect their role in vivo therefore methodologies to examine select IC subtypes in situ should also be considered.

Their needs to be further characterization of newly identified IC markers in different animal models (e.g., mice, rats, guinea pigs, pigs, etc.) and comparisons made to human

tissues. This needs to be extended to pathological models including; spinal cord injury, partial bladder outlet obstruction, diabetes, and neurodegenerative diseases. IC-specific studies could be performed in transgenic models such as Ano1 knockout mice or *c*-kit mutant mice to determine if ICs are indeed responsible for driving detrusor overactivity following a pathological insult. In this way, a more thorough cellular characterization could be performed including histology, electrophysiology, and organ bath studies that can be correlated with cystometric parameters.

Some of the research questions derived from the discussion at the ICI-RS 2013 meeting that warrant addressing are the following:

(1) What is the role of urethral ICs in lower urinary tract dysfunction?
(2) Can bladder IC surface markers be used to differentiate their subtypes and functionality?
(3) Do IC networks propagate regenerative electrical signals in the lamina propria and detrusor layers?
(4) What are the end target tissues for IC networks: urothelium, afferent or efferent nerves, blood vessels or detrusor smooth muscle?
(5) Are novel markers such as Ano1 potential therapeutic targets for the treatment of bladder pathologies?

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Does Our Limited Knowledge of the Mechanisms of Neural Stimulation Limit Its Benefits for Patients With Overactive Bladder? ICI-RS 2013

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Introduction: Neural stimulation has become an established minimally invasive treatment for various lower urinary tract symptoms. The results both short- and long-term are encouraging, however, there is still a lack of knowledge of obvious risk factors, which may affect the outcome of treatment. Although neural stimulation has been embraced by healthcare professionals and patients, the exact mechanism by which neural stimulation works is still unclear. **Discussion:** A condense review of knowledge available on this topic is presented. Several research questions are raised. Outlines of research studies, both clinical and basic science, are suggested. **Conclusions:** Further studies are necessary to understand mechanism of action of neural stimulation and its implications on treatment outcomes. *Neurourol. Urodynam. 33:618–621, 2014.* © 2014 Wiley Periodicals, Inc.

Key words: neural stimulation; overactive bladder

INTRODUCTION

Neural stimulation has become an established minimally invasive treatment for various lower urinary tract symptoms. There are three main neural stimulation methods.

- *Sacral Nerve Stimulation (SNS)* involves applying an electric current produced by the implantable pulse generator, to one of the sacral nerves via an electrode placed through the corresponding sacral foramen.
- *Pudendal Nerve Stimulation (PNS)* involves applying an electric current produced by the implantable pulse generator, to pudendal nerve via an electrode placed in near proximity to the nerve by the ischial-rectal approach
- *Percutaneous Tibial Nerve Stimulation (PTNS)* involves inserting a tiny needle electrode just above the ankle and stimulating a tibial nerve with electric current.

The results both short- and long-term are encouraging, however, there is still a lack of knowledge of obvious risk factors, which may affect the outcome of treatment. Although neural stimulation has been embraced by healthcare professionals and patients, the exact mechanism by which neural stimulation works is still unclear. There are several hypotheses extrapolated from basic science experiments on bladder reflexes and innervation. Specific and relevant models (clinical and animal) for studying the mechanism of neural stimulation are scarce. Improved knowledge of the mechanism(s) of action of neural stimulation could improve outcomes by more appropriate patient selection and by modifying surgical techniques. We undertook a brief review of our clinical and basic science knowledge with a particular emphasis of outlining the deficiency in our understanding of mechanism of action of neural stimulation. Several research questions are raised. Outlines of research studies, both clinical and basic science, are suggested.

WHAT DO WE KNOW FROM THE CLINICAL STUDIES?

- Sacral Nerve Stimulation (SNS) has been used for refractory overactive bladder "dry" and "wet" for more than a decade. Good outcomes have been reported in 70–80% of patients who received a permanent implant.[1] These results have continued for several years of follow-up, however, reoperation rate is high and in the range of 30–40%.[2] Although exact mechanism have not yet fully described, several hypotheses have been proposed. Leng & Chancellor suggested that SNS suppresses urgency and alters bladder function through activation of the pudendal afferents and by turning off supra-spinally mediated overactive voiding by blocking ascending sensory pathway input.[3] Afferent mediated response to rather direct motor nerve stimulation can explain an anal sphincter contraction observed during peripheral nerve evaluation. Mean latency of response was approximately 10 times longer than would be expected from that resulting from direct motor nerve stimulation.[4] It is unclear if the same apply to bladder responses as well. It has been postulated however that SNS modulates afferent pathways, altering spinal and supraspinal circuits, restoring balance between inhibitory and excitatory control systems through a Gate-controlled mechanism.[5] It has also been stated that SNS suppresses detrusor contractions by activation of the bladder-sphincter-bladder reflex. Urine storage reflexes are organized in the spinal cord, whereas voiding

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reflexes are mediated by a spinobulbospinal pathway passing through a coordination center (the pontine micturition center) located in the brainstem.[6] Sacral dorsal root neural stimulation reduces c-fos gene expression and bladder hyperreflexia in spinalized rats, through inhibition of afferent c-fiber activity.[7] This explains some reports of beneficial outcome of SNS on patients with Bladder Pain Syndrome/Interstitial Cystitis.[8] The beneficial effect of SNS on urinary retention/voiding dysfunction is much more difficult to explain. It has been suggested that SNS suppresses detrusor inhibitory interneurons and releases the bladder from an augmented sphincter-bladder reflex at the spinal and supraspinal level.[9]

- Clinical outcomes of SNS depend also on specific stimulation parameters because of the different nerve fibers captured with stimulation. This may elicit some therapeutic advantages. The data are however very scarce and with limited clinical significances.[10,11]
- Pudendal Nerve Stimulation (PNS) has been utilized for overactive bladder (OAB). There are some reports indicating PNS to be superior to SNS. Almost all who failed SNS responded to PNS stimulation (93.2%). Overall, positive PNS response ($\geq$50% improvement) was achieved in 71% of participants.[12] In another study, after temporary stimulation of pudendal nerve or sacral roots, the majority of the patients chose PNS over SNS (13 vs. 4).[13]
- Percutaneous Tibial Nerve Stimulation (PTNS) has been used for OAB and studied by several investigators.[14,15] This ambulatory procedure (1$\times$ week, 30 min) for 6–8 weeks has positive outcomes in 70–80% and the effect persists for several months with booster treatment. The mechanism of action is believed to be similar to acupuncture, through central nervous system neurotransmitter release. This treatment is reserved for patients who failed other conservative treatments and should be considered as experimental treatment because of scarce literature data on outcomes.

HOW DIFFERENT ARE DIFFERENT FORMS OF NEURAL STIMULATION?

The mechanism and site of action of the different forms of neural stimulation and which ones are best suited to treat the different causes of detrusor overactivity or voiding dysfunction is unclear. The type of nerves stimulated by different forms of neural stimulation (usually unilateral) could be a key to their mechanism of action and the form of overactivity and hypersensitivity they are best suited to inhibit. SNS through the S3 foramen, and PNS near the transgluteal or ischial-rectal region, stimulate mixed somatic, and visceral nerves. Low amplitude stimulation (just below the threshold for an anal "wink") is used to capture large somatic Aα- and Aβ- and midsize Aδ-afferents. Midsize B-fibers, which include visceral sympathetic and parasympathic efferents, may also be stimulated but not small C-fibers which have thresholds 50 times greater than Aδ- and B-fibres.[16] Therefore, nociceptive afferents may be less affected by these two forms of neural stimulation than mechanosensitive afferents and visceral efferents which may favor OAB wet. Alternatively, percutaneous posterior tibial nerve stimulation (PPTNS) near the heel or medial malleolus will stimulate mainly somatic nerves and, since 1.5–3 times the threshold to produce extensor flexion (without discomfort) is employed, this may also capture C-fibers and favor OAB-dry.[17]

The outcome is affected by stimulation parameters. High frequency (>20 Hz) stimulation cause detrusor contractions in non-obstructive urinary retention and low frequency (5–10 Hz) stimulation inhibits detrusor activity in OAB. This variable response depends on pelvic efferent activation via the pelvic ganglion and not on adrenergic pathways.[18]

DO LABORATORY ANIMAL STUDIES PROVIDE MORE ANSWERS?

Various animal models have been used to investigate the lower urinary tract (LUT) effects of the different forms of neural stimulation in order to discern their mechanisms of action and improve stimulation techniques for better patient outcomes. However, SNS,[19] PNS,[20] and tibial nerve stimulation (TNS)[17] have only been studied acutely. PNS has been proposed as a method of improving bladder emptying by modulating afferents innervating the urethra. It has the benefit that there are generally positive results in those who fail to respond to SNS and it can be administered transcutaneously using noninvasive surface electrodes. The mechanism of action is still unclear however and long-term studies are lacking in animals and humans. PTNS is the newest treatment route for neural stimulation. It has the advantages that it can be administrated transcutaneously and has a persistent benefit on LUT symptoms following cessation of treatment.[21] In rats, PTNS has a lower efficacy compared to SNS,[22] however, PTNS may be elicited through remodeling of central pathways which would not be evident in acute studies.

The Kanai lab has been successful in using chronic TNS to suppress irradiation-induced detrusor overactivity in mice for at least 3 days after the final round of stimulation. TNS (10 Hz, 2 ms, 15–20 V) is administered (1 hr/day for 6 days), starting 1 week after focal irradiation (10 Gray; 1 Gy = 100 rads) of the bladder—as presented at the 2013 ICI-RS meeting in Bristol, UK. The persistent inhibition of detrusor overactivity can be demonstrated on cystometrograms from decerebrate mice, but not in isolated bladder sheets excised with their associated spinal roots. This suggests that the sites where TNS-induced changes occur are not in the forebrain or periphery, but in the brainstem (among others also location of the pontine micturition center) and spinal cord. Since the beneficial effects of SNS and PNS cease when stimulation stops, these indicate involvement of different mechanisms and sites of action with real-time stimulation of inhibitory neuronal circuits rather than their long-term remodeling as may occur with TNS. Accordingly, this mouse model is well suited to characterize TNS where sustained inhibition will allow in vivo testing of inhibitors to determine the neurotransmitters involved, and in vitro isolation and serial-sectioning of the midbrain and spinal cord to determine neurotransmitter sites of action.

Neurotransmitters are thought to play a role in neural stimulation, which could lead to combination therapies using drugs and electrical stimulation to increase their effectiveness. A number of acute animal studies have demonstrated a putative involvement of glutamate, serotonin, and opioid receptors in the effect of neural stimulation on control or irritated bladders. Glutamate receptors probably are involved in PNS-[23] and PTNS-mediated[24] inhibition of bladder overactivity in cats. Serotonin receptors are also suggested to be involved in PNS-mediated bladder inhibition,[25] however, the serotonin reuptake inhibitor, duloxetine, did not exhibit additive benefits during TNS in cats.[26] Opioid receptors, particularly type μ, were suggested to be involved in the effects of SNS and PNS in normal cat bladders. Together with glutamate receptors, they are also reported to produce synergic effects in TNS of acetic acid irritated bladders.[27] However, the opioid receptor antagonist, naloxone, failed to decrease PNS-induced inhibition of detrusor overactivity in irritated bladders[23] and TNS-induced inhibition of reflex bladder contractions in control cats.[27] Thus, the types of

neurotransmitters and their role in neural stimulation is still unclear but their elucidation may benefit from the use of the chronic TNS mouse model described above.

The hypothesis that nociceptive fibers promote OAB-dry and mechanosensitive ones OAB wet is feasible if second order Aδ-fibers project to the periaqueductal gray region of the midbrain and their third order ones to the pontine micturition center to trigger micturition; while second order C-fibers project to the thalamus and insula and their third order ones to the cortex to signal bladder fullness. To test this hypothesis, work is on-going in the Kanai lab to develop viral constructs that express genetically encoded green and red fluorescent Ca^{2+} indicators driven by promoters unique to mechanosensitive or insensitive fibers. Since these viruses cross synapses, they can be injected into the detrusor to track bladder afferent fiber distribution in the brainstem and cortex. One important consideration is that not all Aδ-fibers are mechanosensitive and not all C-fibers are nociceptive. Approximately half of the bladder afferents are Aδ-fibers of which ~70% are mechanosensitive (80% high thresholds and 20% low threshold or silent requiring sensitization to respond) and 30% are mechanoinsensitive. On the other hand, essentially all C-fibers are high threshold and normally silent. However, half of them are noxious and half can respond to mechanical stimulation when sensitised.[28]

PATIENTS PERSPECTIVE

One pilot study showed that highest scores for perception of a successful outcome and satisfaction with outcome were significantly more common in the 75 min group shared appointment group despite leaving objective measures of success unchanged when compared to office counseling only.[29] Others showed improvements in short-term patient knowledge about SNS using a Patient-Based Educational Video in comparison to manufacturer's video (MV).[30] Only patient's knowledge and no treatment outcome measures have been utilized in this study.

RESEARCH QUESTIONS AND STUDY OUTLINES

- **Basic Science**
  - Do the different forms of neural stimulation preferentially treat different types of bladder overactivity or hypersensitivity?
  - Test the different forms of neural stimulation with animal models of bladder overactivity (e.g., neurogenic, myogenic and mixed) and hypersensitivity (e.g., chemical, radiation, and bacterial cystitis).
  - Are different types of afferent nerves (i.e., mechanosensitive versus nociceptive) responsible for OAB wet and OAB-dry?
  - Inject the bladder wall with pseudorabies virus (PRV) containing genetically encoded green fluorescent protein or red fluorescent protein (e.g., m-Cherry), driven by promoters unique to Aδ- and C-fibers. This would be followed at an appropriate time by removal, serial-sectioning, and histological evaluation of the spinal cord, brain stem and cortex.
  - Can developed refractoriness to neural stimulation be due to remodeling of neural circuits?
  - Perform long-term neural stimulation followed by PRV tracing studies to evaluate remodeling in the periphery and CNS.
- **Clinical Studies**
  - Are there benefits to the combination of neural stimulation and pharmacotherapy in the "naÿve" patients?
    - A randomized controlled trial (RCT), intention-to-treat comparing two group of patients;
      - SNS with or without pharmacotherapy at least 24 months in duration.
      - Outcome measures based on validated questionnaire and objective measures that is, pad test. Urodynamic parameters can be considered.
  - Do different stimulation sites or parameters have clinical significance in the treatment outcomes?
    - RCT comparing patients with unilaterally versus bilaterally implanted electrodes.
      - At least 3 months duration
      - Outcome measures as above
    - RCT comparing patients with S3 versus S4 lead placement
      - At least 3 months duration
      - Outcome measures as above.
    - Difficulties in standardization of precise electrode placing, diversity of human anatomy and electrode position flexibility, RCT trials investigating outcome impact of stimulation parameters, can only be done using the patient as its own control
      - At least 1–3 months duration of each period of stimulation with different parameters
      - Outcome measures as above
  - Does patient knowledge about the mechanism of action of neural stimulation improve outcomes?
    - A randomized controlled trial (RCT) comparing two group of patients
      - Control Group; patients implanted with a standard office counseling
      - Active Group; Patients with extensive pre implant interview with detail explanation of the mechanism of action of neural stimulation
      - At least 24 months duration each period of stimulation with different parameters
      - Outcome measures as above

CONCLUSIONS

It is obvious that our understanding of mechanism of action of different forms of neural stimulation is very superficial and more clinical and animal studies are needed to better comprehend how neural stimulation works. It is unclear if this lack of knowledge translates into the inferior benefits to the patients.

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Urothelial Mucosal Signaling and the Overactive Bladder-ICI-RS 2013

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There is abundant evidence that the lower urinary tract (LUT) mucosal layer is involved both in mechanosensory functions that regulate bladder contractile activity and in urethral sensation. Changes to the mucosa can be associated with a number of bladder pathologies. For example, alterations of the urothelium and underlying lamina propria at both the molecular and structural levels have been reported in both patients and animals associated with disorders such as bladder pain syndrome and diabetic cystopathy. In contrast to the urinary bladder, much less is known about the urothelium/lamina propria of the bladder neck/proximal urethra. There are important gender differences in the outflow region both anatomically and with respect to innervation, hormonal sensitivity, and location of the external urethral sphincter. There is reasonable evidence to support the view that the mucosal signaling pathway in the proximal urethra is important for normal voiding, but it has also been speculated that the proximal urethra can initiate bladder overactivity. When dysfunctional, the proximal urethra may be an interesting target, for example, botulinum toxin injections aiming at eliminating both urgency and incontinence due to detrusor overactivity. *Neurourol. Urodynam. 33:597–601, 2014.*

Key words: bladder nerves; epithelium; lamina propria; neuroendocrine; overactive bladder; sensation; urethra

INTRODUCTION

The urothelium as a part of a mucosal signaling pathway[1] has attracted increasing attention for its potential role in the pathophysiology of the overactive bladder syndrome/detrusor overactivity (= OAB). Most of the lower urinary tract (LUT) afferents are concentrated to the outflow region[2] (Fig. 1), which implies that the urothelium in this part may be of special importance for the generation of both normal and abnormal bladder activity. Since the outflow region in men and women are distinctly different, both anatomically and with respect to innervation and hormonal sensitivity, gender-dependent factors have to be considered when urothelial afferent signaling mechanisms from the urethra are discussed. For example, the female bladder neck is lacking the smooth muscle sphincter structure that serves as a protection against retrograde ejaculation in males (Fig. 2), and also the dense adrenergic innervation seen in the male bladder neck[3,4] (Fig. 3). In addition, the location of the external striated sphincter is different (Fig. 2). In the human female LUT, estrogen receptors are found in squamous epithelia of the trigone and urethra, but not in bladder urothelial tissues[5] (Fig. 4). There is no variation with estrogen status. In contrast, estrogen receptors (both alpha and beta) have been found in the rodent urothelium.[6,7] In the human male LUT, estrogen receptors are found in the urethral epithelium, lamina propria, and periurethral glands.[8]

BLADDER AND URETHRAL EPITHELIUM

A prerequisite for conscious bladder control is adequate sensory input to the central nervous system (CNS), and it is well established that changes in afferent mechanisms may give rise to disturbances of bladder function. Morrison[9] (referring to Nathan)[10] stated that "the sense of filling and the desire to micturate probably depend on afferent endings in the bladder itself, whereas the sensation that micturition is about to happen probably originates from the urethra, as does the sensation that urine is being passed." In addition, a variety of structures play an important role in terms of urethral closure (such as the urethral epithelium, vasculature, and smooth muscle) that are necessary to maintain continence.[11] The urothelium, forms the interface between the urinary space and the underlying lamina propria that contains a dense vasculature as well as connective, nervous, and muscle cells (muscularis mucosae).[1,12,13]

In contrast to the urinary bladder, where the urothelium is composed of at least three layers of epithelial cells, very little has been described regarding the urethral epithelium. There seems to be no apparent difference between the urothelium of the trigone compared to the detrusor.[14] This is in contrast to the proximal urethra where the urothelium transitions to a stratified or columnar epithelium accompanied by a lack of urothelial-specific differentiation markers.[15,16] In terms of the underlying structures that are likely to contribute to function, there are similarities to that of the bladder body. For example, the urethral epithelium is likely to be part of a signaling system involving projections of neuroendocrine cells, interstitial cells and sensory nerve endings. There is speculation that these urethral-neuroendocrine cells (sometimes termed paraneurons)[17] could release mediators such as serotonin, which via activation of adjacent sensory nerves can stimulate urethral

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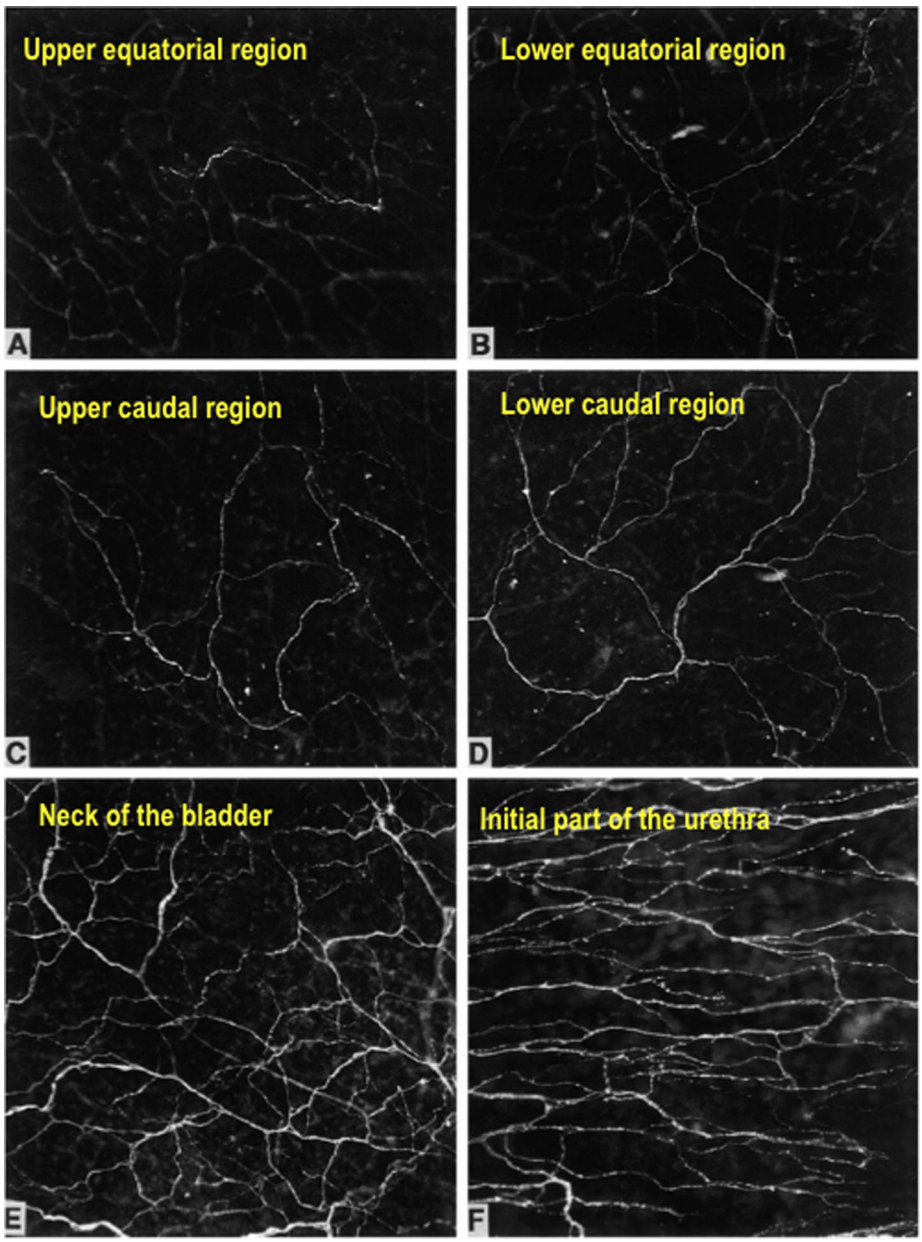

Fig. 1. Whole mounts of rat bladder stained with calcitonin-gene related peptide (CGRP) antibodies from different regions.[2]

reflexes. Such types of cells are not unlike that in other types of epithelia, such as the trachea, where a cell type termed brush cells have been described which are likely chemo-receptive and make contact with nearby nerve fibers.[18] There is also a rich vascular network that lies beneath and a dense distribution of nerves throughout the bladder neck and initial part of the urethra[2,19] where the nerves form a plexus adjacent to the urothelial lining.

There is some evidence that the urothelium in the region of the urethra may play a role in continence and sensation. The mucosal pathway (often referred to as a sensory web)[1,12,20] within the proximal urethra also involves a cascade of epithelial inhibitory and stimulatory transmitters/mediators. Release of these factors may be involved in a complex transduction scheme underlying the activation of bladder nerves to play a prominent role in sensation. In addition, it has been suggested that symptoms of pain that arise from the lower urinary tract may originate from the bladder neck and proximal urethra.[21] The bladder neck and proximal urethral contain the largest density of nerves[2,19] and the epithelial cells that line the surface exhibit "neuronal-like" properties including expression of proteins sensitive to chemical and physical stimuli.[20] The proximity of afferent nerves to the epithelium suggests that epithelial cells could be targets for transmitters released from nerves and/or that chemicals released by epithelial cells influence afferent nerve excitability. Thus, urethral epithelial-neural interactions (via release of mediators) may lead to a "urethral instability" that can influence storage and voiding reflexes and result in symptoms including urgency and pain.

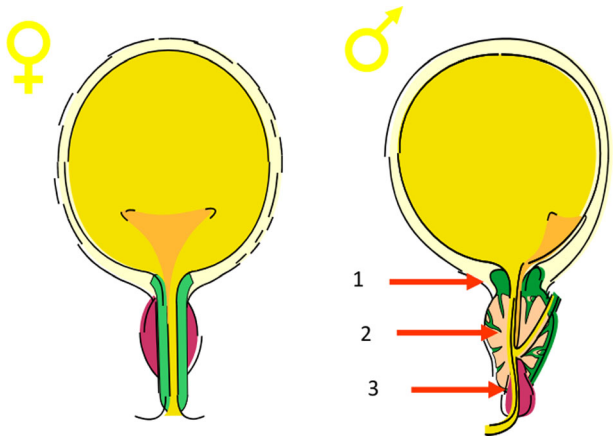

Fig. 2. Anatomical gender difference. Green color: urethral smooth muscle; red color: striated urethral muscle. Note the presence of an internal smooth muscle sphincter in the male (arrow 1: "sexual" sphincter, preventing retrograde ejaculation), the location of the external urethral sphincter in the male (arrow 3), and the presence of the prostate (arrow 2).

URETHRAL SENSATION

Sensory signals from the urethra arise during urine flow and a fundamental question arises whether urethral stretch and/or shear stress caused by urine flow contribute to sensory signals. By analogy with the bladder, lateral stretch of the urothelium releases transmitters such as ATP[22] as does equivalent relaxation.[23] This therefore suggests that there is a dynamic component to stretch in the urothelium, which in the urethra would derive from variations in urine flow.

Sensory signals from urethral afferents can initiate detrusor contractions and also maintain this contraction by positive feedback through continuous flow of urine through the urethra.[24,25] If this urethro-vesical reflex is eliminated by urethral anesthesia, the detrusor contraction fails and excessive straining is needed to achieve bladder evacuation in multiple spurts.[25]

There is evidence for slowly and rapidly adapting afferents to steady urethral flow,[26] indicating that both the rate and duration of urine flow may be sensed. However, the afferent nerve population is not homogenous, and more than one population of sacral interneurones seem to be involved.[27] This further suggests that discharge may depend on both the urethral flow itself and the rate of change of flow.

The mechanism of afferent activation remains unclear, but by analogy with the bladder itself one possibility is that shear stress on the urethral urothelium induces the release of transmitter molecules to mediate the effect. In other tubular structures, such as blood vessels shear-stress releases mediator molecules such as ATP from the endothelium[28] and a hydrodynamic analysis would indicate a similar phenomenon is feasible in the urethra. In the left-anterior descending coronary artery (diameter 3.5–4.0 mm) peak diastolic velocity is about 22 ml/sec.[29,30] A similar maximum urine flow rate (25 ml/min) occurs in normal adult men and women through a urethra that varies in diameter from 1 to 12 mm.[31,32] These two variables are key determinants of the shear stress on a vessel wall[33] and their similar values in blood vessels and the

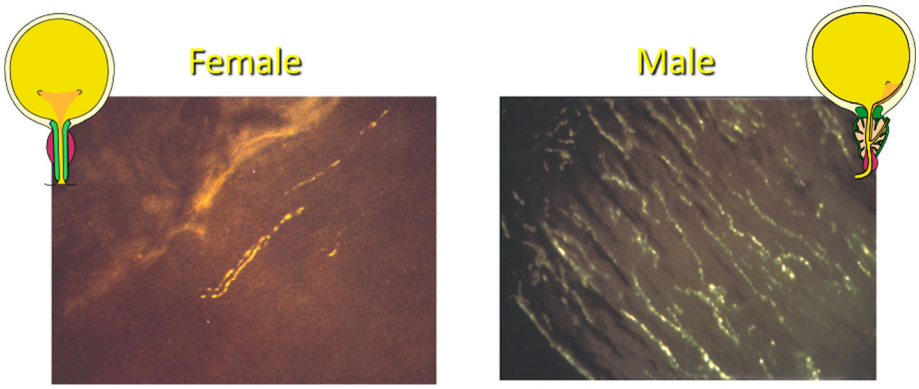

Fig. 3. Gender difference on the distribution of noradrenergic nerves in the human bladder neck.[51]

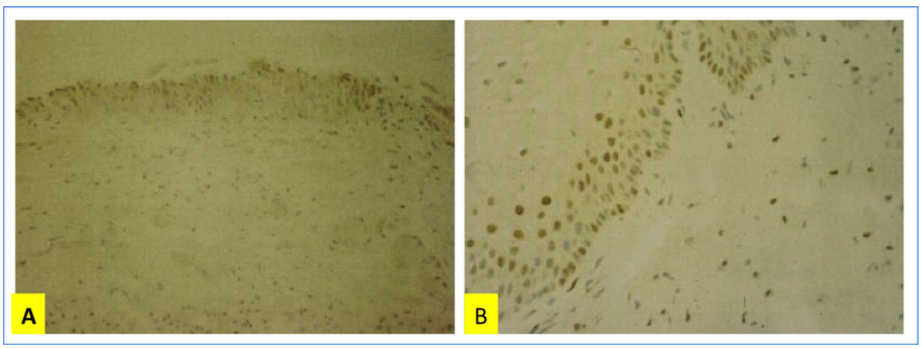

Fig. 4. Estrogen receptor (ER) distribution in the female LUT. The bladder urothelium (**A**) is ER-negative, whereas the urethra is ER-positive.[5]

urethral walls and will suggest that flow-mediated shear stress could indeed evoke mediator release from the urethral wall.

The evidence for release of mediators from urethral urothelium is weaker than in the bladder. Histological evidence shows that urothelial cells secrete mucus, similar to that of other cells with microvilli and small granules and which can be interpreted as evidence of secretion.[34] Endogenous nitric oxide (NO) synthase is present in urethral urothelium[35] and nitric oxide may be released during stress either from lamina propria nerves or from the urothelium itself.[36,26] Local prostaglandin release may influence urethral function as exogenous PGE2 reduces urethral resistance.[37] Evidence for stress-activated release of ATP or acetylcholine from urethral urothelium, as occurs in bladder urothelium, is lacking.

THE PROXIMAL URETHRAL MUCOSA AS A POSSIBLE INITIATOR OF OVERACTIVE BLADDER

The pathophysiology of lower urinary tract (LUT) symptoms, including the overactive bladder syndrome, is multifactorial[38] and may involve various LUT structures. As mentioned previously, the distal urethral epithelium is composed of stratified squamous cells, arranged in longitudinal folds, which variably becomes transitional as the bladder neck is approached. The epithelium is supported by a loose lamina propria, consisting of collagen fibrils and elastic fibers, arranged both circularly and longitudinally, and a rich network of blood vessels and nerves.[13]

The epithelium together with the interstitial cells and the suburothelial afferent nerves have been suggested to form a functional unit ("the mucosal signaling pathway") able to initiate a bladder contraction.[39] Gabella and Davis,[2] studying the distribution of bladder afferent nerves in the rat bladder, found afferent axons distributed over four distinct areas: at the base of the epithelium, inside the epithelium, on blood vessels (both arteries and veins) and along muscle bundles. In the lamina propria, all the afferent axons, except the perivascular ones, were found either inside the epithelium or in a subepithelial plexus very close to the basal surface of the epithelium. The plexus was thickest in the neck of the bladder and in the proximal portion of the urethra, and it became progressively less dense in the adjacent regions. The cranial region of the bladder had no afferent axons.

The combination of a urothelial mechanosensory function[1] and the distribution of afferent nerves have focused interest on the proximal urethra as an initiator of detrusor overactivity, and several previous studies support such a possibility. Tanagho and Miller[40] observed that urethral relaxation occurs a number of seconds before detrusor contraction under normal circumstances, and Low,[41] demonstrated a similar sequence occurring in female patients with idiopathic detrusor overactivity. The presence of urethral pressure variations during filling cystometry (urethral instability) has been well documented by various investigators, and said to be responsible for the sensation of urgency and for incontinence.[42] Mechanical stimulation of the urethra, especially the flow of urine, may facilitate the micturition reflex as mentioned above, and reflexes between the urethra and bladder play an integral role in the neural control of the lower urinary tract.[25] Studies in animals have demonstrated the basis for an excitatory urethra-to-bladder reflex, showing that sensory nerves in the wall of the urethra fire in response to urethral urine flow and electrical stimulation, and that this activity initiates bladder contractions in the quiescent bladder and augments ongoing contractions in the active bladder. Mazieres et al.[43] showed in cats that electrical stimulation of pudendal urethral sensory nerves can activate bladder efferent neurons and initiate detrusor contractions. Urethra-to-bladder reflexes thus seem to be mediated by afferent inputs traveling through the pudendal nerve to the sacral spinal cord[44] and brain.[45,46] Attempts to identify an excitatory urethra-to bladder reflex in humans using urethral urine flow have been difficult,[25,47] and produced results either opposing or supporting the presence of such a reflex. Gustafson et al. stimulated the prostatic urethra electrically via a catheter-based electrode in five men with complete spinal cord injury and succeeded in initiating detrusor contractions in four of five individuals when the bladder volume was sufficiently large.[48] These results demonstrate in humans the presence of a volume-dependent excitatory bladder reflex mediated by urethral afferent nerve fibers. The neural circuitry was suggested to exist in the lumbosacral spinal cord not requiring a brainstem pathway.

There is thus reasonable evidence supporting the view that mucosal signaling pathways in the proximal urethra are important for normal voiding, and that when dysfunctional can be an interesting target for example, botulinum toxin.[49,50] The ability of botulinum toxin injected into the urethral suburothelial space to block afferent nerves involved in urgency and DO remains to be established. The urethral mucosal signaling pathway is as yet not fully explored and promises to be an interesting and rewarding research field many research questions can be raised.

RESEARCH QUESTIONS

(1) Which are the mechanisms by which bladder and urethral epithelial cells, when exposed to different sensory "inputs" (mechanical, chemical), evokes different afferent discharges? In what ways are such mechanisms influenced by pathology?
(2) Can urethral flow, or rate of change of flow, differentially effect discharge from urethral afferents?
(3) Is urethral sensitivity to urine flow is modulated by exogenous agents (ATP, acetylcholine)?
(4) If the proximal urethra is a generator of detrusor overactivity—then how does treatments (blocking nerves in the suburothelial space) affect such overactivity?

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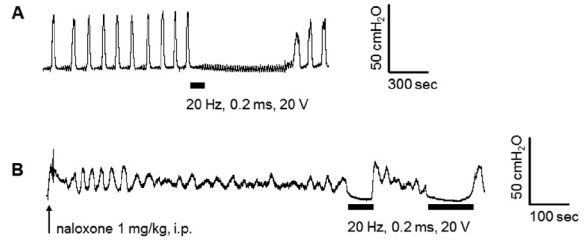
Effect of the hippocampal electrical stimulation on isovolumetric contraction in urethane anesthetized rats



Effect of the hippocampal electrical stimulation on isovolumetric contraction in urethane anesthetized rats. A. Inhibitory effect of 20-Hz-hippocampal electrical stimulation on isovolumetric bladder contraction. B. Effect of naloxone on hippocampal inhibition of isovolumetric contraction. The duration of hippocampal stimulation is indicated by the black bar under the bladder pressure trace.

Source of Funding: Supported by the NIH under grants DK-068566, DK-090006 and DK-091253.

MP17-17
SENSORY EVOKED CORTICAL POTENTIALS OF THE LOWER URINARY TRACT IN HEALTHY MEN

Martina D. Liechti, Stephanie Knuepfer, Flavia Gregorini, Martin Schubert, Armin Curt, Thomas M. Kessler, Ulrich Mehnert, Zürich, Switzerland*

INTRODUCTION AND OBJECTIVES: Sensory evoked cortical potentials (SEPs) are established diagnostic tools to objectively assess sensory function within different areas of the body. Such tool can be of diagnostic value for the assessment of lower urinary tract (LUT) afferent properties. Recently we demonstrated reliable recording of SEPs from different LUT sites in healthy females. However, still little is known from male LUT. Aim of the study was to evaluate the feasibility and reliability of SEP recording following stimulation at different localizations in male LUT.

METHODS: Ten healthy men (age:19-37 years, height: 1.85±0.05 meters) were measured twice (intervall:1-3 weeks). During electroencephalographic (EEG) recording, one-millisecond, repetitive (0.5Hz) square wave stimuli were applied via a special transurethral 8F catheter to urethral (distal, membranous, proximal) and endovesical stimulation sites (trigone, bladder wall). The bladder was drained and refilled with 60mL contrast medium after each stimulation cycle.

EEG data were filtered (0.5-30Hz band-pass plus 50Hz notch filter), segmented and averaged per subject and localization. Focus of analysis was cortical component N1 recorded from the Cz electrode referenced to Fz (10/20 system). Statistical tests comprised analysis of variance (ANOVA), intraclass correlation coefficients (ICC), linear regressions, and paired t-tests. Values are given as mean ± standard deviation.

RESULTS: LUT SEPs could be detected with a prominent N1 and P2 and a small P1 component in Cz-Fz signal. N1 latencies were most reliable (ICC:0.61-0.77) and localization-specific (bladder wall:127.1±18.6ms, trigone:133.5±14.2ms; urethra: proximal:130.8±17.9ms, membranous:126.1±32.2ms, distal:113.0±14.6ms). N1P2 amplitudes were highly reproducible (ICC:0.89-0.96) within subjects, but greatly varied between subjects.

N1 latencies resulting from stimulation at the bladder wall, membranous and distal urethra, demonstrated a significant (all $p<0.01$) negative correlation with subject age.

CONCLUSIONS: SEPs can be reliably recorded following electrical stimulation at different LUT sites in men. Variability of N1P2 amplitudes between subjects seems to be higher compared to within subjects, which can be explained by the individual electrical and pain perception. Location specific differences in N1 latencies might reflect different local afferent innervation, i.e. different fiber density and quality. LUT SEPs provide promising measures to monitor treatment outcome and functional recovery. Further investigations are warranted in larger cohorts and patients.

Source of Funding: Swiss National Science Foundation

MP17-18
BIDIRECTIONAL COMMUNICATION BETWEEN AFFERENT NEURONS AND UROTHELIAL CELLS IN THE MOUSE URINARY BLADDER

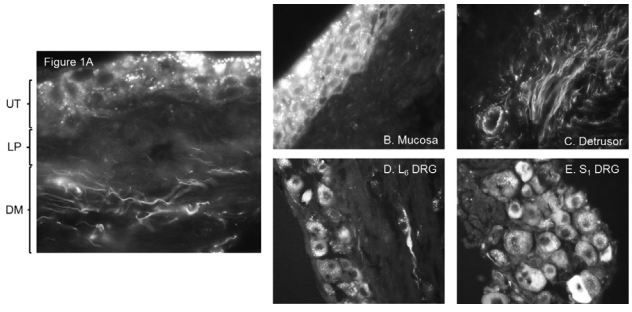
Youko Ikeda, Irina Zabbarova, Anthony Kanai, Pittsburgh, PA*

INTRODUCTION AND OBJECTIVES: It has been widely hypothesized that urothelial cells communicate unidirectionally with afferent terminals to signal changes in the bladder lumen milieu. However, direct interactions between afferents and urothelial cells have not been clearly demonstrated. Our aim was to examine these interactions using pseudorabies virus (PRV) with the genetically-encoded Ca^{2+} probe, GCaMP to localize its fluorescence in the mouse urinary bladder.

METHODS: For dorsal root ganglia (DRG)-PRV injections, adult female C57Bl/10 mice were anesthetized and a hemilaminectomy performed at the L5-L6 vertebrae level to expose L6-S1 DRG. 2 μl of PVR468-GCaMP3 (10^6 pfu/ml) was slowly injected into each DRG using a 1.0 mm glass micropipette with a 10 μm beveled tip. The muscle and skin were sutured, animals allowed to recover and used for experiments after 2-3 days. For sham controls, sterile saline was injected in place of viral vector. In additional experiments, 10 μl of PRV was injected into the wall of the descending colon or tail muscle. Isolated DRG or whole bladder sheets were fixed, sectioned and examined for GCaMP fluorescence.

RESULTS: Following DRG-PRV injections GCaMP labeling was found in afferent nerves in the detrusor (DM) and within the urothelium (UT), but not the lamina propria (LP) (figure 1A). Labeling in the UT appeared punctate or contained inside vesicles, suggesting there may be urothelial uptake of GCaMP or PRV from afferents. In colon and tail injections, a similar GCaMP expression pattern was found compared to DRG injections (B and C). Colon/tail injected animals also had dense expression in L6/S1 DRG, demonstrating that these structures have shared afferent innervation with the bladder (D and E). Fluorescence was not detected in the DRG or bladders of sham control animals (not shown).

CONCLUSIONS: The established concept has been that urothelial cells signal to suburothelial afferents to 'sense' changes within the bladder lumen milieu. We have demonstrated that communication between afferents and urothelial cells is bidirectional, where afferents can also signal to urothelial cells. Changes in the dynamics of urothelial-afferent communication may underlie sensory disorders of the lower urinary tract.



Source of Funding: R01DK071085 to A. Kanai.

MP17-19
MIRABEGRON SELECTIVELY INHIBITS NOCICEPTIVE BLADDER AFFERENTS

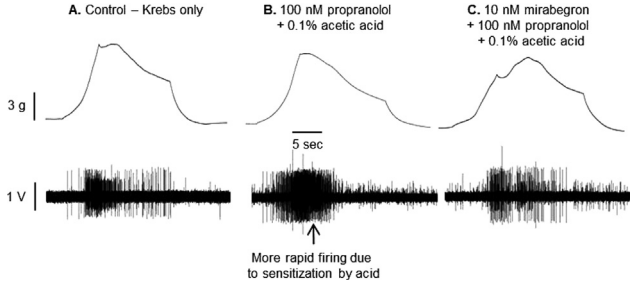
Youko Ikeda, Irina Zabbarova, Lori Birder, Anthony Kanai, Pittsburgh, PA*

INTRODUCTION AND OBJECTIVES: The mechanism of action of the β_3-adrenoceptor agonist, mirabegron is believed to occur *via* relaxation of detrusor smooth muscle during filling and dampening of afferent nerves. In this study, the effect of mirabegron on urinary bladder pelvic and hypogastric afferent nerve firing was evaluated on normal and acetic acid sensitized rat bladders.

METHODS: Female Sprague-Dawley rats were used for *in vitro* bladder-pelvic spinal nerve (L6-S1) and lumbar splanchnic (hypogastric) nerve recordings. Isolated bladder sheets with associated nerves were connected to a tension transducer and nerves were passed into adjacent oil recording chambers. The bladder was stretched *via* a stepper motor to evoke mechanosensitive firing. Mirabegron (10 nM - 1 μM) was added to the superfusion fluid in the presence of 100 nM propranolol to block β_1- and β_2-adrenoceptors.

RESULTS: Mirabegron did not alter stretch-evoked afferent activity at all concentrations tested (not shown). Addition of 0.1% acetic acid to the superfusion fluid elicited large amplitude intrinsic detrusor contractions, activated spontaneous afferent firing and enhanced stretch-evoked firing rates (A and B). At low doses of mirabegron ($<$ 100 nM), enhancement of stretch-evoked afferent activity was reduced to that during control conditions (C). The baseline tension and tension generated from stretches was not significantly affected at concentrations $\leq$100 nM, demonstrating that mirabegron did not promote relaxation at pharmacologically relevant concentrations.

CONCLUSIONS: During bladder filling, β_3-adrenoceptor activation mediated through the hypogastric nerve is thought to relax the detrusor to reduce filling pressure. However, we have demonstrated that mirabegron preferentially acts on nociceptive afferents over mechanosensitive afferents and detrusor smooth muscle at clinically relevant concentrations ($<$ 25 nM). These data suggest that there are functional β_3-adrenoceptors on afferent neurons (most likely nociceptive C-fibers) and that these are not activated during bladder filling under physiological conditions. However, in pathology these silent fibers can be activated and mirabegron inhibits their activity (*i.e.,* nociception). This may be a reason for the efficacy of mirabegron to reduce lower urinary tract symptoms without significantly affecting voiding function.



Source of Funding: Grant from Astellas Pharma (A. Kanai and L. Birder) and DoD (SC100100, A.Kanai).

MP17-20
CHARACTERIZATION OF FUNCTIONAL BLADDER REGENERATION IN MICE

Mona Zarifpour, Jason Sandberg, Mehran Abolbashari, Manasi Vadhavkar, Sneha Kelkar, Aaron Mohs, Frank Marini, Karl−Erik Andersson, George Christ, Winston-Salem, NC*

INTRODUCTION AND OBJECTIVES: Previous studies have established that subtotal cystectomy (removal of 60-70% of the bladder) provides a valid and unique model of mammalian organ regeneration (1,2). The goal of this study was to characterize this phenomenon in mice, a species that can be genetically engineered to investigate a diverse array of molecular alterations of functional bladder regeneration, including specific cell−based fluorescence imaging technologies.

METHODS: Female C57 black mice underwent STC, and at 4 and 12 weeks post−STC, bladder regeneration was assessed via cystometry and ex vivo pharmacologic organ bath studies. Histology was also performed to measure bladder wall thickness and the composition/architecture of the bladder wall.

RESULTS: We observed a time−dependent increase in bladder capacity following STC, such that 12 weeks post−STC, the sizes of regenerated bladders and micturition volumes were indistinguishable from those of age−matched controls (Fig. 1A, C). Bladders emptied completely at all time points studied (i.e., no increases in residual volume), consistent with functional bladder regeneration. There were no significant differences in bladder wall thickness (Fig. 1B) from controls, or in the percentage of smooth muscle in the detrusor layer (not shown). Contraction to pharmacological activation and electrical field stimulation were present in isolated tissue strips from regenerating bladders but were lower than controls at all time points. Initial cell and tissue transplantation studies using fluorescently labeled mice (i.e., transfer of green fluorescence protein bone marrow into red fluorescent protein animals) revealed a participation and contribution of bone marrow derived populations toward functional bladder regeneration.

CONCLUSIONS: This study extends our investigation of mammalian bladder regeneration from rats to mice. Establishing a validated model for the study of de novo organ regeneration in mice will allow for further characterization of this phenomenon through detailed investigation of its molecular and cellular basis.

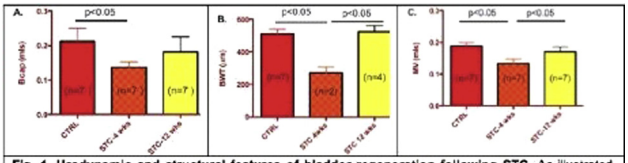


Fig. 1. Urodynamic and structural features of bladder regeneration following STC. As illustrated, consistent with previous observations in rats, we observed a **complete functional regeneration** within 12 weeks of ≈60% STC (subtotal cystectomy). This is reflected by the fact that bladder capacity (BC), micturition volume (MV) and bladder wall thickness (BWT) are all indistinguishable between mice 12 weeks post-STC and Age Matched Control animals (CTRL). This was a time-dependent regenerative response, as CTRL animals are significantly greater than animals at 4 weeks post-STC with respect to all 3 parameters.

Source of Funding: NIH P20 Grant DK097806

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NEUROSCIENCE 2014

Presentation Abstract

Program#/Poster#:	736.13/OO8
Presentation Title:	p75 Receptor inhibition decreases spinal cord injury-induced bladder damage
Location:	WCC Hall A-C
Presentation time:	Wednesday, Nov 19, 2014, 8:00 AM - 9:00 AM
Presenter at Poster:	Wed, Nov. 19, 2014, 8:00 AM - 9:00 AM
Topic:	++E.04.f. Gastrointestinal, renal/urinary, and reproductive regulation
Authors:	***I. V. ZABBAROVA**[1], S. O. YOON[3], W. C. DE GROAT[2], L. A. BIRDER[1], A. J. KANAI[1]; [1]Med., [2]Pharmacol. and chemical biology, Univ. of Pittsburgh, Pittsburgh, PA; [3]Mol. and Cell. Biochem., Ohio State Univ., Columbus, OH
Abstract:	**Objective:** Spinal cord injury (SCI) at a supralumbar level is accompanied by a number of changes in bladder function including loss of voluntary micturition control, detrusor hyperreflexia and bladder-sphincter dyssynergia; this is followed by detrusor hyperactivity and hypertrophy. It was also demonstrated that SCI leads to a rapid disruption of the urothelial permeability barrier followed by urothelial hyperplasia due to mechanisms that are not well understood. p75 is a neurotrophin receptor which binds uncleaved pro-NGF and pro-BDNF (triggering apoptosis) and later the mature active proteins (promoting cell growth). It has recently been shown that SCI rapidly increases p75 expression and neurotrophin levels in the bladder. We hypothesized that pro-neurotrophin binding to p75 urothelial receptors promote the loss of umbrella cells and barrier function early after injury. We have assessed changes in the bladder wall following SCI and benefits of a p75 inhibitor, LM11A-31, in preventing these changes twenty four hours post injury. **Methods:** Female 4-5 weeks old C57Bl10 mice were gavaged with 100 mg/kg

LM11A-31 diluted in 100 µl of water one day prior to SCI (T8-T9) which was performed with or without urinary diversion. Following surgery, mice were treated with an analgesic and prophylactic antibiotic and their bladders manually expressed twice a day. Twenty four hours post surgery, animals were anesthetized, perfused, and their bladders isolated for histology. **Results:** Control bladder sections demonstrated a urothelium consisting of three to four cellular layers, distinct interstitial cells and a thick detrusor. Twenty four hours following SCI, the urothelial layer was disrupted and the muscle atrophied. Urinary diversion prevented the loss of the urothelium but not muscle thinning which could be prevented by LM11A-31. **Conclusion:** Acute SCI results in urothelial loss and bladder atrophy; the latter was prevented by p75 receptor blockade but not by urinary diversion. This suggests that pro-neurotrophins may be released into the urine which is blocked by diversion. Ongoing studies are investigating the relative levels of pro-neurotrophins released by the bladder and kidneys following SCI and the potential benefits of p75 inhibition in chronic SCI.

Disclosures: **I.V. Zabbarova:** None. **S.O. Yoon:** None. **W.C. de Groat:** None. **L.A. Birder:** None. **A.J. Kanai:** None.

Keyword (s): SPINAL CORD INJURY

NERVE GROWTH FACTOR

BLADDER

Support: NIH Grant P01DK09324

DOD SCI100100

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NEUROSCIENCE 2014

Presentation Abstract

Program#/Poster#:	736.22/OO17
Presentation Title:	Viral probes injected into the mouse tail are expressed in dorsal root ganglia and the bladder urothelium; a possible mechanism for the development of interstitial cystitis
Location:	WCC Hall A-C
Presentation time:	Wednesday, Nov 19, 2014, 9:00 AM -10:00 AM
Presenter at Poster:	Wed, Nov. 19, 2014, 9:00 AM - 10:00 AM
Topic:	++E.04.f. Gastrointestinal, renal/urinary, and reproductive regulation
Authors:	***Y. IKEDA**[1], I. V. ZABBAROVA[2], W. C. DE GROAT[3], L. A. BIRDER[2], A. J. KANAI[2]; [1]Sch. of Med., [2]Med., [3]Pharmacol. and Chem. Biol., Univ. of Pittsburgh, Pittsburgh, PA
Abstract:	**Objective:** Pseudorabies virus (PRV) tail injections in rodents have been previously demonstrated to induce neurogenic inflammation and enhance nociceptive responses in the urinary bladder without signs of infection. However, the neurons involved in PRV-induced cross-sensitization have not been fully characterized. Therefore, we utilized PRV expressing a green fluorescent Ca^{2+} sensitive fusion protein (GCaMP) to trace the affected neurons in the bladder following tail injections. **Method:** C57Bl/10 mice were anesthetized and injected with 10 µl of PRV-GCaMP (Becker strain, 10^8 plaque forming units/ml) into the abductor caudalis dorsalis (tail muscle) with a Hamilton syringe and 30 gauge needle. One week following injections, mice were anesthetized with 2.5% avertin (2,2,2-tribromoethanol) and perfused *via* the heart with oxygenated Krebs solution. The urinary bladder and L_6 and S_1 dorsal root ganglia (DRG) were removed, placed in 4% paraformaldehyde and

then incubation in 30% sucrose. Fixed tissues were mounted in optimal cutting temperature medium and frozen on dry ice. These were sectioned 6-8 μm thick, mounted on slides and the presence of GCaMP ($\lambda_{ex}488/\lambda_{em}509$) examined using fluorescence microscopy. **Results:** The urinary bladder and L_6 and S_1 DRG were examined for GCaMP labeling one week post-injection. GCaMP is a Ca^{2+} sensor; with fixation the cytosol is flooded with Ca^{2+} that binds to GCaMP locking it in the fluorescent state. Within the bladder wall, the urothelium and afferent nerves were distinctly labeled. Labeling in the urothelium appeared punctate and localized within the cells, suggesting there was direct PRV infection. Labeling was also found in sensory neurons within the L_6 and S_1 DRG that send projections the urinary bladder. **Conclusion:** Cross-sensitization is thought to be a potential cause for interstitial cystitis/painful bladder syndrome and viral infections may represent a sub-set of patients afflicted by this condition. In this study, we demonstrate that PRV can cross-infect different organs and that viral proteins can affect not only the sensory neurons but also the urothelial layer. As the urothelium can directly modulate the activity of innervating sensory neurons, its dysregulation may compound the effects of PRV-induced neurogenic inflammation and nociception.

Disclosures: **Y. Ikeda:** None. **I.V. Zabbarova:** None. **W.C. de Groat:** None. **L.A. Birder:** None. **A.J. Kanai:** None.

Keyword (s): VIRUS

SENSORY NEURONS

BLADDER

Support: R01-DK071085

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DOD SCI100100